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Some volatile compounds in New Zealand Cheddar cheese and their possible significance in flavour formation

III. Time of first appearance of volatile carbonyl compounds during ripening

By R. J. HARVEY* and J. R. L. WALKER
The Dairy Research Institute (N.Z.), Palmerston North, New Zealand

(*Received* 20 *August* 1959)

Summary. Two Cheddar cheeses were analysed for aldehydes and methyl ketones at intervals during the ripening period. One-day-old cheese was found to contain acetaldehyde and acetone, together with traces of butan-2-one and pentan-2-one. As the cheese matured heptan-2-one, nonan-2-one and undecan-2-one appeared, and the concentrations of all these compounds progressively increased.

In the first paper of this series (Walker & Harvey, 1959) the isolation of various carbonyl compounds from steam distillates of mature Cheddar cheese was described, and the possible significance of these compounds in cheese flavour was discussed.

The present paper reports the results of a study of the development of acetaldehyde and methyl ketones during the ripening of New Zealand Cheddar cheese.

MATERIALS AND METHODS

Materials

Cheese. Several 7 lb cheeses were made from one batch of curd in the Institute's experimental factory. The cheeses were stored at 55 °F until they were waxed at 14 days, and subsequently at 45 °F. This is in keeping with normal commercial practice in New Zealand. A second batch of cheese was made 3 months later, and was waxed and stored as above.

Analyses for carbonyl compounds were carried out at 1 day, and at 2 and 4 weeks on both batches of cheese, and thereafter at 4-weekly intervals. Each time an entire cheese was taken and at the time of each analysis samples of the cheese were presented to a panel of judges for assessment of flavour and stage of maturity.

Reagents. The various carbonyl compounds used as reference substances were obtained from L. Light and Co., England.

Methods

Estimation of carbonyl compounds. A 500 g sample of the cheese to be analysed was coarsely shredded and placed in a 2-litre bolt-head flask containing 500 ml of distilled water, sufficient to form a thick slurry. This mixture was then steam-distilled,

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and the distillate led directly into 100 ml of 2,4-DNP (2,4-DNP denotes '2,4-dinitrophenyl' throughout this paper) hydrazine reagent (1 g/l. in 2N-HCl). This technique guarded against any possible loss of the more volatile compounds. When 250 ml of distillate had been collected, the flask containing distillate and reagent was placed in a water-bath at 45 °C for 2 h. The 2,4-DNP hydrazones were then extracted with chloroform (20 ml, followed by two successive 10 ml quantities). The chloroform extract was evaporated down to approximately 2 ml by means of a stream of warm air, quantitatively transferred to a glass-stoppered graduated test tube, and the volume made up to 5 ml with chloroform. A 0·02 ml portion of this solution was then applied to a Whatman no. 1 chromatogram paper. Paper chromatography of the mixed 2,4-DNP hydrazones followed the method of Lynne, Steele & Staple (1956). The individual 2,4-DNP hydrazones were identified and estimated using the spectrophotometric methods described by the present authors in previous papers (Walker & Harvey, 1959; Harvey, 1960). By this means an estimate of the levels of the various carbonyl compounds in the distillate was obtained.

Recovery of carbonyls from cheese. In order to relate the concentration of each carbonyl compound in the distillate to its actual concentration in the cheese it was necessary to determine the percentage recovery of each compound when added to a cheese slurry. This was done as follows. About 1 kg of cheese was shredded and accurately divided into two 500 g portions. The first portion was distilled, and the carbonyls in the distillate estimated as described above. Known quantities of various methyl ketones were added to the second portion and thoroughly dispersed in the cheese slurry before distillation. Recovery rates were then calculated from the differences between the two determinations. The results are presented in Table 1. In order to provide a check on any possible effect that the age of the cheese may have on the recovery rate of added methyl ketones, several recovery experiments were carried out using cheeses of different ages (ranging from 1 to 8 months). No significant differences in recovery rates were observed from the various cheeses, so there is no doubt that the data presented in Fig. 1 represent a real increase in carbonyl content as the cheese ripens.

Table 1. *Percentage recovery rates of various methyl ketones added to a cheese slurry*

	Recovery rate (±2%)		
Added ketone	Young cheese (1 month old)	Mature cheese (8 months old)	Mean recovery rate
Acetone	73	75	74
Butan-2-one	68	68	68
Pentan-2-one	64	62	63
Heptan-2-one	67	65	66
Nonan-2-one	40	41	41
Undecan-2-one	11	12	12

RESULTS

The experimental cheeses ripened normally, and at the conclusion of the experiment were judged to be of good quality, with a typical mature Cheddar flavour.

It may be seen from Fig. 1 and Table 2 that the 1-day-old cheeses were found to

contain acetaldehyde and the following methyl ketones: acetone, butan-2-one and pentan-2-one. After 2 weeks the concentration of acetone was found to have diminished, but thereafter it gradually increased again. A trace amount of heptan-2-one was first detected after 2 weeks. After 8–12 weeks the first definite sign of a mature Cheddar cheese flavour became apparent to the panel of judges and the intensity of the Cheddar flavour slowly increased as the cheeses matured. This increase of

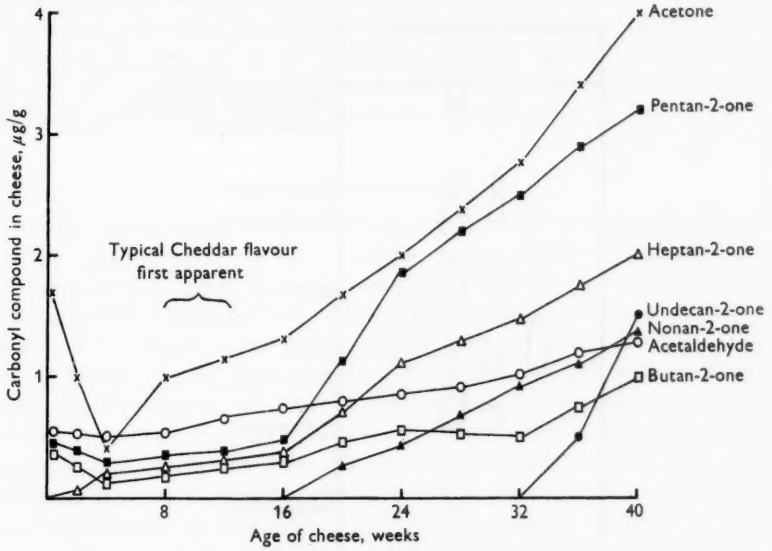

Fig. 1. Formation of various carbonyl compounds during ripening of Cheddar cheese.

Table 2. *Identification and time of appearance of carbonyl compounds in Cheddar cheese*

Compound	R_{but} value*	Absorption maxima, mμ: Ethanol	Ethanolic KOH: Major	Ethanolic KOH: Subsidiary	First detected (time after manufacture)
Acetaldehyde	0·45	357	426	500–510 (inflexion)	1 day
Acetone	0·70	361	434	525	1 day
Butan-2-one	1·00	360	434	528	1 day
Pentan-2-one	1·25	360	432	526	1 day
Heptan-2-one	1·70	361	433	527	2–4 weeks
Nonan-2-one	2·25	364	433	534	20–24 weeks
Undecan-2-one	2·75	364	434	534	36 weeks

* R_{but} = movement relative to butan-2-one DNP hydrazone.

Cheddar flavour was paralleled by a progressive increase in the levels of pentan-2-one, heptan-2-one and nonan-2-one as shown in Fig. 1. Apart from butan-2-one, all the methyl ketones formed during ripening possessed an odd number of carbon atoms in their chains; this suggests that they may be produced by some type of decarboxylation reaction from the even-numbered aliphatic fatty acids present in the milk fat.

In order to provide a check on the possible formation of carbonyl compounds due to heating during the steam-distillation process, an additional experiment was conducted using a previously described technique (Walker, 1959), modified to permit the use of low-temperature and low-pressure conditions. A stream of nitrogen was bubbled for 24 h through a slurry of mature cheese maintained at 45 °C and 30 mm Hg pressure. The issuing gas was then led into a solution of 2,4-DNP hydrazine

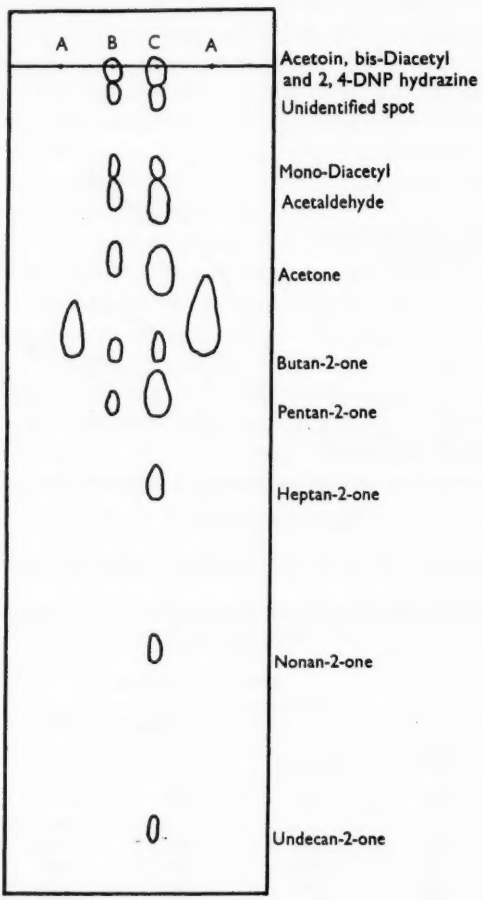

Fig. 2. Paper chromatogram of 2,4-DNP hydrazones of carbonyl compounds from Cheddar cheese distillates. Solvent system: heptane—2-phenoxyethanol. A, Authentic 2,4-DNP hydrazone of butan-2-one; B, distillate from 2-week-old cheese; C, distillate from 36-week-old cheese.

reagent to trap any carbonyl compounds. The following carbonyl compounds were recovered: acetaldehyde, acetone, butan-2-one, pentan-2-one, heptan-2-one and traces of nonan-2-one. It is thus most probable that the methyl ketones are present in mature Cheddar cheese as such and are not merely present as the result of decomposition during the steam-distillation process.

The actual results reported here (Fig. 1 and Table 2) represent the average of experiments on two batches of cheese manufactured at different times, and although slight variations occurred, they did not affect the over-all trends which were apparent during the ripening process. It should be emphasized that the main object of this work was to establish the time of first appearance of each particular carbonyl compound. Independent confirmation of the above findings was gained from other similar experiments with young (1–14 days) and mature (9–12 months) cheeses. Cheddar cheese, even when manufactured under close control, is variable particularly with respect to flavour, but the considerable difference in carbonyl pattern between young and mature cheese was observed in all experiments and is well illustrated by the chromatogram in Fig. 2 and by the results shown in Fig. 1.

DISCUSSION

The results presented in this paper show that a range of methyl ketones may be isolated by steam distillation from Cheddar cheese. These compounds increase in concentration as the cheese matures, particularly after the typical Cheddar flavour becomes apparent. The possibility of their being artifacts generated during the heat treatment of the milk fat has been considered, since Patton & Tharp (1959) and Bassett & Harper (1958) have presented evidence that this may possibly occur. However, the experiments of Patton & Tharp (1959) were conducted at the abnormally high temperature of 200 °C, and their results are therefore not strictly comparable with those presented here. Bassett & Harper (1958) found that the heating of a slurry of Blue Vein cheese at 100 °C for 10 min increased the yield of heptan-2-one and nonan-2-one, no doubt due to the decarboxylation of β-keto acids. In the present experiments it seems unlikely that the methyl ketones were formed in this way since comparable amounts of the same substances were identified in experiments not involving high-temperature distillation [similar findings have also been recorded by Patton, Wong & Forss (1958) and Day & Keeney (1958)]. Furthermore, the carbonyl pattern of the steam distillates changed in a regular way as the cheese matured. Little variation in carbonyl pattern would be anticipated if the substances were artifacts formed as a result of the heat treatment of the milk fat.

The evidence therefore indicates that methyl ketones are present as such in the Cheddar cheese, and are not artifacts produced during steam distillation. They are, moreover, linked with cheese flavour by the previous finding (Walker & Harvey, 1959; Walker, 1959) that the 'cheesy' aroma of a neutralized distillate from the Cheddar cheese is destroyed when the carbonyl compounds are removed by the addition of 2,4-DNP hydrazine reagent.

The fact that a group of closely related compounds in cheese shows an increase in concentration as the flavour of the cheese develops cannot, by itself, be taken as conclusive evidence that such compounds are responsible for all or part of the flavour. However, the results presented in the previous papers of this series (Walker & Harvey, 1959; Walker, 1959) provide strong evidence that methyl ketones are responsible for an important part, but not all, of the Cheddar cheese aroma. The present results can therefore be considered as further confirmation of this hypothesis.

The authors wish to thank Dr H. R. Whitehead and Dr W. A. McGillivray for much helpful criticism and advice, and Mr P. L. McLaughlin for technical assistance.

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With 4 plates

The distribution of bacteria in New Zealand Cheddar cheese

By C. G. RAMMELL
Wallaceville Dairy Laboratory, Department of Agriculture, Wellington, New Zealand

(*Received* 25 *March* 1960)

Summary. The distribution and development of bacteria during the maturing of New Zealand Cheddar cheese have been studied using stained sections of cheese. The starter streptococci usually occur singly and in small groups throughout the mass of the cheese, their distribution on a microscopic scale being very irregular. In contrast, the bacteria developing later form discrete microcolonies, most of which are associated with the curd junctions. Instead of adding lactobacilli to milk, smaller numbers may be added to the dry curd before cheddaring or after milling. Some observations on the physical structure of cheese are made.

This work has extended over two years and was begun in the hope that it might provide evidence of an association between specific bacteria and 'tallowy discoloration' in Cheddar cheese. Early results, however, revealed some interesting features to which reference could not be found in the literature. The scope of the work was therefore extended to a general investigation of the distribution and development of bacteria in cheese. Most of the work was done with New Zealand Cheddar cheese, although other types of cheese were examined for comparison.

METHODS

Preliminary experiments

Initially, sections were cut from the interior of mature New Zealand Cheddar cheese of normal make from four different factories. The sections were cut 6 μ thick by the Histology Section, Animal Research Station, Wallaceville, using standard wax-embedding methods, and were then stained using carbol thionin or Gram's stain.

Main experiments

Cheese manufacture. Most of the sections were cut from 10-lb 'loaf' and 80-lb 'export' Cheddar cheese made from pasteurized milk at Waihakeke Dairy Factory, New Zealand, by Mr A. Sellars, Dairy Instructor.

One 10-lb cheese was made from curd which after milling and salting was well mixed with 300 ml of 1·7 % (w/v) aqueous solution of Congo red at 150–170 °F. By this means, the surfaces of the milled curd were coloured red.

Three 80-lb cheese were made from milk to which a clotted culture of *Lactobacillus* sp. 25·2 (from N.Z. Cheddar cheese; Sherwood, 1939) was added at different stages of the cheese-making process. The first of these cheese was made from 800 gal of

milk to which 2 gal of the clotted culture were added with the starter. This is equivalent to 1·6 pints per 80-lb cheese. One of the resulting cheese was used for sampling. The second cheese was obtained from another vat by thoroughly mixing 3 pints of the sample culture with sufficient curd at drying to make two 80-lb cheese, one of which was used for sampling. The third cheese was obtained by thoroughly mixing 1·5 pints of the same culture with sufficient milled curd, immediately before salting, to make two 80-lb cheese, one of which was used for sampling.

Sampling of cheese. Samples were taken with a trier vertically through the top of the 10-lb cheese, and horizontally through the sides of the 80-lb cheese. All samples were completely submerged in 10% (v/v) formalin for at least 24 h before cutting.

Section cutting, mounting and staining. Sections were cut in the Dairy Laboratory using a Unicam freezing microtome. Disks, 2 mm thick, of the formolized cheese, were first cut from the centre of the cheese plug and then submerged in distilled water for 10 min before sectioning. The instrument was set to cut at 6 μ, but the thickness of the resulting sections was not always constant at 6 μ. Sections were floated on 50% (v/v) rectified spirits, transferred to distilled water, and finally mounted on slides coated with a very thin film of glycerine jelly (Carleton, 1926).

With four of the cheese, triplicate sections of the sample plugs were cut, stained and examined. The disks of cheese for sectioning were cut from the plug approximately 4, 7 and 9 cm from the surface of the cheese.

For differentiation of the fat and casein, sections were stained with Herxheimer's solution (Cowdry, 1948) and counterstained with Loeffler's methylene blue. The bacteria were not always clearly seen in these sections.

For examination of the bacteria, the fat was first removed by standard histological methods and the sections then stained with carbol thionin, followed by differentiation with 95% (v/v) alcohol.

Examination of sections. Stained sections were scanned on the moving stage of a microscope, using the 10× objective and 8 × 2·25 compound eyepiece. Colonies of bacteria were readily seen at this magnification; under ideal conditions, single cells could sometimes be distinguished. For detailed examination, the 40× objective was used.

Photographs were taken of some of the first sections cut and prints were made at a magnification of 15–30 diameters. On these prints colonies appeared as dark areas (Pls. 1, 2). Unfortunately, the dark areas did not always represent colonies of bacteria, thus making the following procedure necessary. While the author scanned the section on the microscope stage giving the position of any colonies seen, an assistant plotted these colonies on a sheet of thin tracing paper placed over the corresponding photographic print. With experience, it was possible to correlate the position of the colonies as seen under the microscope with the dark areas on the photograph. The tracing was then transferred to a sheet of drawing paper (Fig. 1, see p. 350). The technique was not applied to all the sections because of the considerable time and eyestrain involved.

RESULTS

Physical structure of cheese

In the fat-free sections stained with thionin the curd junctions were readily visible as fine lines (Pls. 1, 2). In the sections stained to show the fat, the curd junctions evidently contained less fat than the enclosed curd: the density of the casein in the junctions was correspondingly higher. In certain cases, milled curd junctions could be distinguished by their relationship to other curd junctions (Pl. 2). This relationship where a junction is seen with other junctions at 'right' angles to it, and on both sides of it, is a result of the mechanical milling of the cheddared curd. Except where this characteristic distribution occurs, the milled curd junctions cannot be distinguished with certainty, although they often tend to be more prominent. This was evident in sections of the 'loaf' cheese made from stained milled curd, where these junctions were well differentiated as red lines.

Although the curd junctions were usually seen as quite fine lines, 'casein masses' were occasionally seen where the casein density was high and the fat concentration low (Pls. 1, 2). 'Loose networks', of spongy appearance and low casein density, were also sometimes seen. The significance of these structures was not studied. The 'casein masses' may, of course, simply be longitudinal sections through part of a curd particle boundary.

Similar pictures were obtained with Cheddar cheese of Australian and Canadian make, and with such other types as Cheshire, Gouda, Edam, and Double Gloucester cheese all made overseas. The precise ages of these cheese were unknown, but all were over 4 months. As was to be expected, differences were noted in the ratio of fat to casein, and in the size and distribution of the fat globules. Sections of Dunlop cheese contained many 'casein masses' and shortish, interrupted junctions, the broken nature of which was presumably due to the manufacturing method which involves breaking and re-forming the curd several times (Davis, 1955).

In some sections of Cheddar cheese, and especially in the sections of Gouda cheese that were examined, curious annular structures were seen (Pl. 3A). These were more commonly formed on the curd junctions, but were scattered throughout the Gouda. Since the annuli sometimes contained small globules of fat, it seems possible that they were formed by shrinkage of the curd after the release of fat into the whey. Sometimes, however, the structures reacted differently from the rest of the curd during differentiation with 95% (v/v) alcohol, suggesting a chemical difference. They were not apparently associated with bacteria.

Distribution of starter streptococci

The starter streptococci were distributed throughout the whole of the curd, both on and off the junctions. Generally the cells occurred singly or in pairs, but a few short chains were sometimes seen. High concentrations or colonies of streptococci were only occasionally seen. This was presumably due to the cessation of active streptococcal growth soon after the firm curd was formed; that is, active growth of starter streptococci occurred during the early stages of the cheesemaking process when the milk and curd were 'fluid'. Little evidence was obtained of streptococcal

development in the cheese after it was removed from the press, although innumerable starter streptococci having a similar distribution as in young cheese were often still seen in cheese 6–9 months old.

The distribution of streptococci on a microscopic scale was very irregular. The results of an attempt to examine this distribution in thirty-six adjacent fields are given in Table 1. The fifteen fields containing no streptococci were all part of a curd particle section itself containing no streptococci.

Table 1. *Distribution of streptococci in a section of Cheddar cheese 2 days old*

(Cell counts made on thirty-six adjacent fields, 1000× magnification.)

Fields ...	1–4	5–8	9–12	13–16	17–20	21–24	25–28	29–32	33–36
	50	0	0	0	10	65	34	65	55
	0	0	0	0	64	36	87	134	58
	0	0	0	0	27	63	86	144	102
	0	0	0	0	72	89	132	46	73

Table 2. *Distribution of bacterial colonies in sections cut from two Cheddar cheese throughout ripening*

Cheese no. 1				Cheese no. 2			
	No. of colonies in relation to curd junctions				No. of colonies in relation to curd junctions		
Age in days	On	Off	Total	Age in days	On	Off	Total
2	0	0	0	1	0	0	0
9	0	0	0	2	5	0	5
17	0	1	1	5	1	0	1
20	0	3	3	7	2	0	2
35	1	2	3	12	1	0	1
49	2	1	3	14	9	0	9
60	7	1	8	16	2	0	2
84	2	2	4	19	4	0	4
98	5	0	5	27	3	0	3
113	15	3	18	33	59	14	73
168	9	5	14	40	17	2	19
190	13	3	16	54	3	0	3
211	13	5	18	68	8	2	10
232	2	1	3	82	2	0	2
253	11	19	30	98	10	3	13
268	11	15	26	113	39	7	46
293	5	1	6	138	6	3	9
323	7	2	9	168	7	6	13
346	18	4	22	191	8	0	8
377	17	10	27	222	4	1	5
				243	4	1	5
Totals	138	78	216		194	39	233

Characteristics of other bacteria

Distribution of colonies. In contrast to the streptococci, bacteria developing later usually formed well-defined colonies, which had a peculiar distribution in maturing cheese. In many sections, the majority of the colonies lay in intimate contact with curd junctions. Pls. 1 and 2 show typical distributions where the number of colonies

is high. Of the seventy-four separate colonies present in the section shown in Pl. 1, sixty-eight were associated with curd junctions.

Table 2 gives the results obtained from two maturing 80-lb cheese of normal manufacture. These results are not corrected for small variations in size of the sections. Salient features of these distributions are: the wide variations in the number of colonies in individual sections; the comparatively early appearance of colonies in cheese no. 2; and the proportion of colonies intimately associated with the curd junctions. This last feature was affected by the density of junctions on any given section. Since the density was not known until after the sections were cut and examined, this factor presumably had a random effect. Notwithstanding, no gross differences in the density of curd junctions were noted.

Table 3. *Distribution of bacterial colonies in experimental Cheddar cheese during ripening*

(Triplicate sections, each 1·4 cm² approx.)

Age, days	No. of colonies in each of the three sections (totals in parentheses) lying				
	On milled curd junctions	On other curd junctions	Off curd junctions	In loose networks	Total
11	3, 0, 3 (6)	1, 0, 0 (1)	1, 1, 2 (4)	0, 0, 0 (0)	5, 1, 5 (11)
18	10, 4, 3 (17)	2, 2, 4 (8)	3, 0, 2 (5)	0, 0, 23 (23)	15, 6, 32 (53)
25	3, 8, 10 (21)	1, 1, 1 (3)	1, 2, 6 (9)	0, 0, 7 (7)	5, 11, 24 (40)
33	6, 1, 7 (14)	2, 1, 0 (3)	3, 3, 2 (8)	0, 0, 1 (1)	11, 5, 10 (26)
39	1, 2, 4 (7)	1, 8, 1 (10)	3, 8, 7 (18)	3, 4, 1 (8)	8, 22, 13 (43)
46	4, 4, 2 (10)	4, 1, 12 (17)	5, 3, 2 (10)	0, 0, 2 (2)	13, 8, 18 (39)
60	6, 4, 9 (19)	12, 4, 0 (16)	4, 1, 3 (8)	6, 0, 0 (6)	28, 9, 12 (49)
74	3, 1, 4 (8)	1, 3, 1 (5)	3, 7, 3 (13)	0, 0, 0 (0)	7, 11, 8 (26)
88	4, 0, 6 (10)	2, 1, 4 (7)	4, 2, 6 (12)	0, 0, 0 (0)	10, 3, 16 (29)
102	2, 8, 11 (21)	5, 1, 0 (6)	5, 2, 3 (10)	0, 0, 0 (0)	12, 11, 14 (37)
119	3, 1, 4 (8)	2, 1, 14 (17)	2, 1, 3 (6)	0, 0, 0 (0)	7, 3, 21 (31)
144	7, 1, 3 (11)	3, 4, 3 (10)	2, 4, 3 (9)	1, 0, 0 (1)	13, 9, 9 (31)
180	5, 4, 0 (9)	4, 3, 3 (10)	1, 2, 3 (6)	0, 0, 0 (0)	10, 9, 6 (25)
Totals	161	113	118	48	440

Because the milled curd junctions could not be differentiated in the two 80-lb cheese, the proportion of colonies associated with them could not be determined. Table 3 gives the results obtained from the 10-lb cheese in which the milled curd junctions were coloured red. The results are not corrected for the variations in size of the sections. It must be emphasized that the red junctions were readily seen by the naked eye during cutting of this cheese. Some sections contained many milled curd junctions, whilst others contained few or none. The sections examined, and recorded in Table 3, were chosen subjectively as having approximately the same density of milled curd junctions, but no objective measurements were made on the lengths of the junctions. The areas of these sections were estimated by projection of the slides onto squared paper. The mean area was 1·44 cm² with a standard deviation of 0·24 cm². The standard deviation of the mean sectional areas of triplicate sections was 0·23 cm². Correction of the number of colonies to a standard sectional area did not appreciably affect the variation between triplicate sections, the cumulative index of dispersion being reduced only from 91·6 to 78·1. The corrected results are illustrated in Fig. 2. The variation between triplicate sections was too great for there

to be much significance in the variation of total colony count with time. The results again show the high proportion of colonies intimately associated with the curd junctions. The proportion of colonies lying on the milled curd junctions was surprisingly high.

Colonies often occurred in groups associated with the same curd junctions, and individual bacteria in these colonies were usually indistinguishable morphologically (Pl. 3B). It appears likely that many of these colonies were derived from the same mother cells which became separated during the later cheese-making processes.

Table 4 gives the distribution of colonies in the three cheese containing experimentally added lactobacilli. The examination of these cheese showed that, even when the lactobacilli were originally present in large numbers throughout the cheese milk, the majority of the colonies developed along the curd junctions. However, in general, a slightly higher proportion was associated with the curd junctions when the lactobacilli were added to the curd. Some of the colonies undoubtedly would not be derived from the added lactobacilli, but such colonies would be in the minority, and probably of the same magnitude in all three cheese. The ability of the added lactobacilli to grow on the milled curd junctions, despite the initially high salt concentration, was also shown.

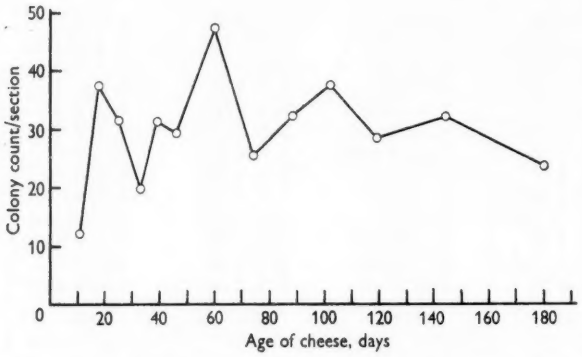

Fig. 2. Variation with time of total colony count in triplicate sections of Cheddar cheese: individual colony counts corrected to standard sectional area of 1·44 cm².

Structure and development of colonies. Individual bacterial cells, other than those associated with colonies, could not be distinguished with certainty in any of the young cheese. The colonies, however, varied considerably in size ranging up to about 130 μ in diameter, with a probable average in mature cheese of 50–70 μ. Single bacteria may have been missed during the examination of sections, but the numbers so missed must be insignificant compared with the numbers present in the recorded colonies. Simple calculations of the number of cells present in a spherical colony of 60 μ diameter, for example, might range up to 10^5, depending on individual size, and on closeness of packing.

The number and size of the colonies in a given section of cheese should enable the original level of infection of the cheese to be calculated. Such a calculation would, of necessity, be very approximate owing to the variations in colony size, colony count, and time of onset of colony development. Taking the results given in Table 3,

for example, the calculated number of mother cells in a gram of the original cheese was approximately 1300. This calculation was based on the four following assumptions: all bacteria that were going to develop had formed recognizable colonies by the time the cheese was 33 days old; average sectional colony count was 11·2; all colonies were 60 μ in diameter (this was taken as the effective thickness of the sections); mean sectional area was 1·41 cm².

Table 4. *Distribution of bacterial colonies in experimental ripening Cheddar cheese containing added lactobacilli*

(Triplicate sections, each 1·4 cm² approx.)

	Colonies/section when lactobacilli added to								
	Cheese milk			Dry curd			Milled curd		
	On	Off		On	Off		On	Off	
Age, days	Curd junctions		Total	Curd junctions		Total	Curd junctions		Total
1	29	2	31	53	2	55	23	1	24
	42	0	42	19	4	23	2	1	3
	38	2	40	20	2	22	8	0	8
	109	4	113	92	8	100	33	2	35
3	*	*	*	84	11	95	33	3	36
	60	7	67	84	3	87	24	3	27
	47	6	53	61	7	68	53	10	63
	—	—	—	229	21	250	110	16	126
6	72	12	84	28	2	30	123	5	128
	55	12	67	44	2	46	*	*	*
	122	16	138	27	0	27	35	1	36
	249	40	289	99	4	103	—	—	—
10	102	25	127	66	4	70	62	6	68
	54	7	61	105	4	109	33	6	39
	59	7	66	235	9	244	41	0	41
	215	39	254	406	17	423	136	12	148
272	97	5	102	71	17	88	152	13	165
	98	20	118	71	8	79	53	4	57
	45	10	55	64	11	75	34	10	44
	240	35	275	206	36	242	239	27	266

* Owing to an accident, results not available for these two sections.

The colonies were often approximately circular, even when their growth was apparently restricted by the surrounding 'casein' (Pl. 4). Since no significant differences were noted in the shape of the colonies irrespective of the plane in which the cheese was cut, it was assumed that most of these colonies were approximately spherical. In other instances, the colonies followed 'lines of weakness' in the cheese.

The morphology of the individual bacteria, especially small coccobacilli and true micrococci, was not always easy to distinguish owing to their compactness. In sections of cheese containing added lactobacilli, the morphological variation seen in bacterial and colony types suggested that even the same bacterial strain may not always produce identical colonies in the cheese. No orderly progression of morpho-

logical types was noted during the maturing of any of the cheese. Neither was any relationship noted between morphological types and their actual distribution within the curd.

DISCUSSION

During the latter stages of this investigation, the results of similar work on a more restricted scale were reported by Dean, Berridge & Mabbitt (1959). These workers used mainly freezing microtome techniques: embedding in wax was avoided in order 'to minimize the risk of removal, during the dehydrating procedures, of any organisms which might not be firmly fixed to the casein'. Presumably, the fat was not removed from the frozen sections for similar reasons. Likewise, Dawson & Feagan (1957), in examining the distribution of starter streptococci in curd, stained the frozen sections directly by Newman's method without first removing the fat. Work in this laboratory, however, showed that the bacteria were most clearly differentiated by using standard bacteriological stains after the fat had been removed from individual sections with xylene. The standard histological processes involved appeared to remove few, if any, of the bacteria. This was shown by examining consecutive sections cut from the same piece of cheese, and stained with and without prior fat removal. The thickness of the sections in relation to the size of the bacterial colonies often made it possible to cut two consecutive sections having approximately the same distribution of bacterial colonies.

The use of enlarged photographs of stained sections of cheese proved particularly rewarding. It is difficult to obtain a complete picture of the distribution of colonies in sections of cheese simply by examining them under the microscope. By using photographs coupled with tracings on which the individual colonies were plotted after identification as such by detailed examination under the microscope, a true picture of the distribution of all colonies in the section was readily obtained. But even this picture is only two-dimensional. By the use of photographs of stained, serial sections of cheese, it should be possible to build up a complete three-dimensional model of the distribution of colonies in cheese. Because of other commitments, this has not been attempted in this laboratory. The method may of course be well suited to the study of certain aspects of the physical structure of cheese.

As discussed by Johns & Cole (1959) and others, it was generally assumed until recently that the lactobacilli did not appreciably increase in numbers until after the streptococcal counts had declined somewhat. Using selective cultural methods, Mabbitt & Zielinska (1956), and Naylor & Sharpe (1958), have obtained evidence that the lactobacilli 'grow slowly and continuously from the beginning of the ripening period'. The present investigation has shed further light on this matter by demonstrating that, in Cheddar and probably some other 'hard' cheese too, the development of bacteria, other than starter streptococci, must be considered as the sum effect of the development of discrete colonies. To the author's knowledge, this fact, although perhaps accepted tacitly by other workers, has not entered greatly into published discussions on the uneven distribution of bacteria in Cheddar cheese. It is, of course, one of the reasons for this unevenness. Mother cells in the fluid milk may be evenly distributed but probably sparsely in the case of pasteurized milk, whilst infection during the later cheese-making processes after the curd has formed will

certainly not be even. To these must now be added the uneven development of individual bacterial colonies, which will depend upon the species and strain of mother cell, its growth phase, and its physico-chemical environment.

There seems to be no reason why the growth of lactobacilli and similar organisms should not begin at pressing time or even earlier, especially if the bacteria are already in the logarithmic phase. That active lactobacilli can multiply in cheese less than a day old was clearly shown by examination of cheese containing experimentally added lactobacilli, many well-developed colonies of which appeared when only a day old. During the cheddaring and later cheese-making processes, any young colonies might easily become divided, and so form several separate colonies all derived from the same mother cells. Evidence supporting this reasoning was obtained from some of the sections. Observations made during this work suggested that lactobacilli may be added more efficiently to the milled curd than to the cheese milk, less culture being required to obtain a similar result. This may also apply to the addition of other bacteria to cheese.

The number of colonies found in sections of New Zealand Cheddar cheese was originally thought to be surprisingly large in view of the presumed low initial infection of the pasteurized cheese milk. Since the gross movement of bacteria in Cheddar cheese once out of the press was most unlikely, it was assumed that the heavy initial infection arose from post-pasteurization contamination in the plant.

These results appear to be in marked contrast to those of Dean *et al.* (1959) who reported that colonies were rarely seen, and then only in cracks or holes. They examined five Cheddar cheese, four of which were manufactured from raw milk. Unfortunately, however, the actual numbers of colonies seen and sections examined were not stated. Although colonies were often seen in N.Z. Cheddar cheese following 'lines of weakness', in holes, cracks, etc., such colonies were in the minority, there being little evidence that 'many organisms can only grow in the crevices' of this cheese.

The association of the majority of the bacterial colonies with the curd junctions is a significant finding. There seem to be two possible explanations for this distribution: either, (*a*) the bacterial mother cells were originally present on the outside boundaries of the curd particles to a far greater extent than in the interior—i.e. infection occurred after the curd was cut, or (*b*) conditions on or near the curd junctions were more favourable to bacterial growth.

With the three cheese containing experimentally added lactobacilli, the finding that most of the colonies were associated with the curd junctions in each case favoured explanation (*b*) or a combination of (*a*) and (*b*). Because of their lower fat density, and correspondingly higher casein density, the curd junctions probably afforded a better environment for the development of colonies. No evidence was obtained that the bacteria tended to congregate at the fat droplet surface to any greater extent than that which would be expected to occur by chance in a medium containing about 35 % fat. In a few cases, there was a suspicion that colonies actually avoided droplets of fat.

Work in this laboratory indicates that the milled curd junctions especially may differ chemically in respects other than the ratio of casein to fat. The salt distribution is heterogenous for at least 12–24 h after salting, and the high salt concentration in

the vicinity of the milled curd junctions during this period changes the distribution of certain protein and other constituents (unpublished results). Such changes may favour the development of bacteria in these areas.

The valued assistance of Miss Sheila Stuart in much of this work is gratefully acknowledged. Photographs and line drawings were prepared by Mr A. Bryant. Dr H. R. Whitehead kindly supplied the large amounts of culture of lactobacilli used in the manufacture of some of the experimental cheese. The constant encouragement of Dr G. M. Moir, Dairy Chemist, throughout this work is much appreciated.

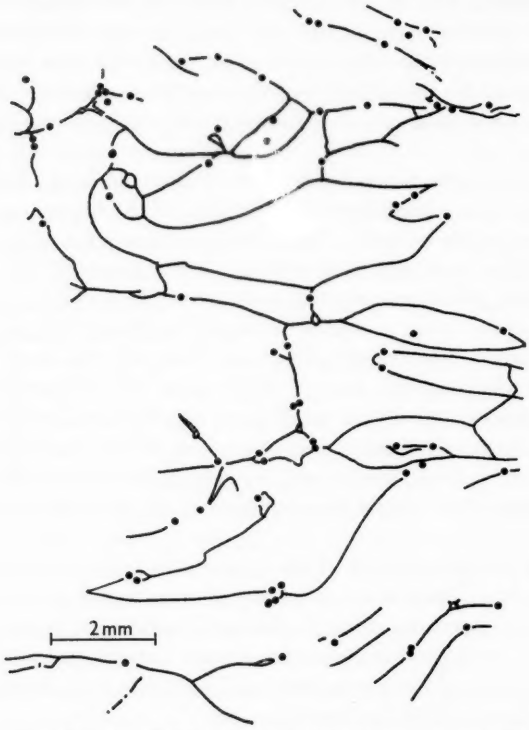

Fig. 1. Tracing, prepared as described in text, of section of cheese shown in Pl. 1. Positions of bacterial colonies are shown by dark spheres—enlarged for illustrative purposes. Only sufficient of the curd junctions are included to enable correlation with the actual colonies seen in Pl. 1.

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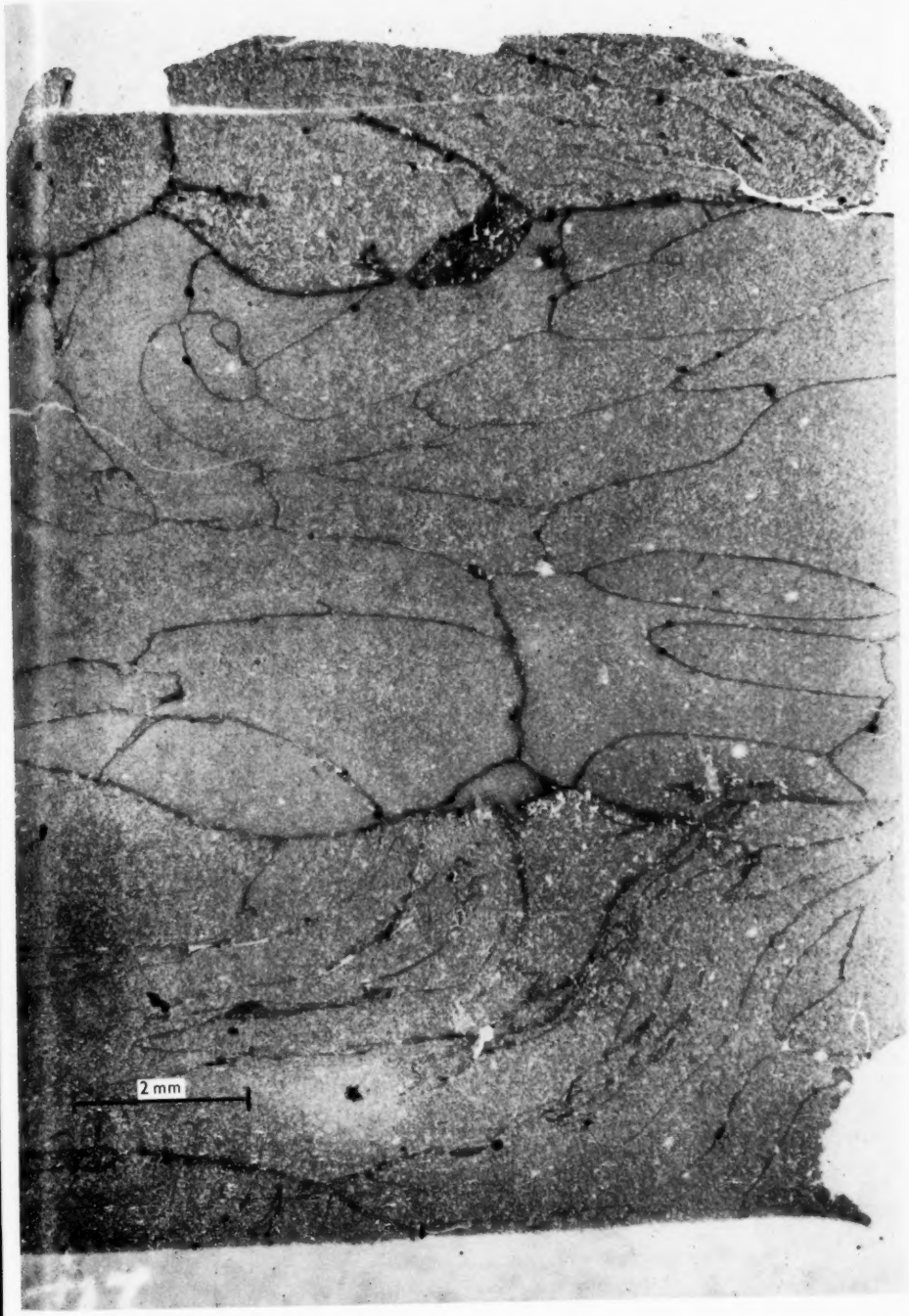

C. G. RAMMELL

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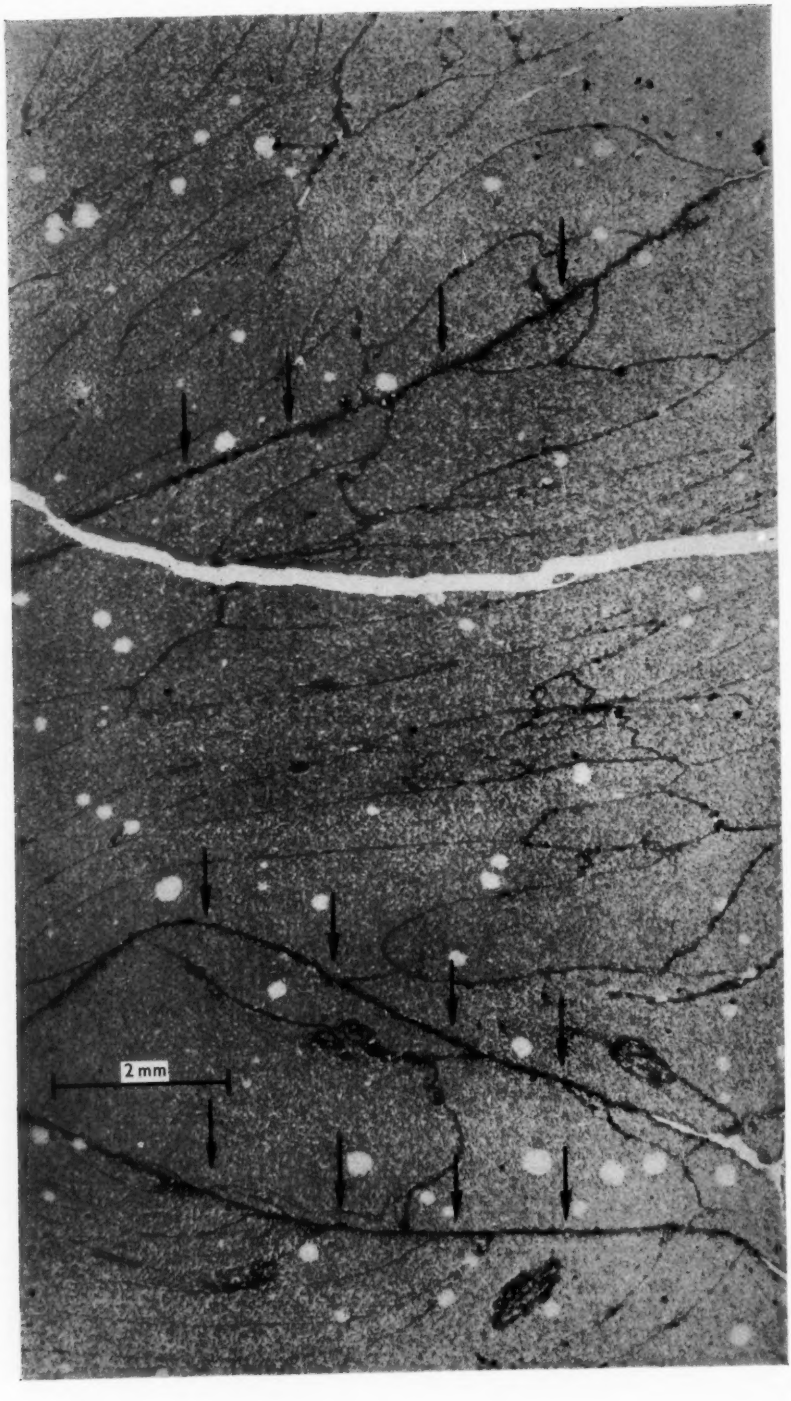

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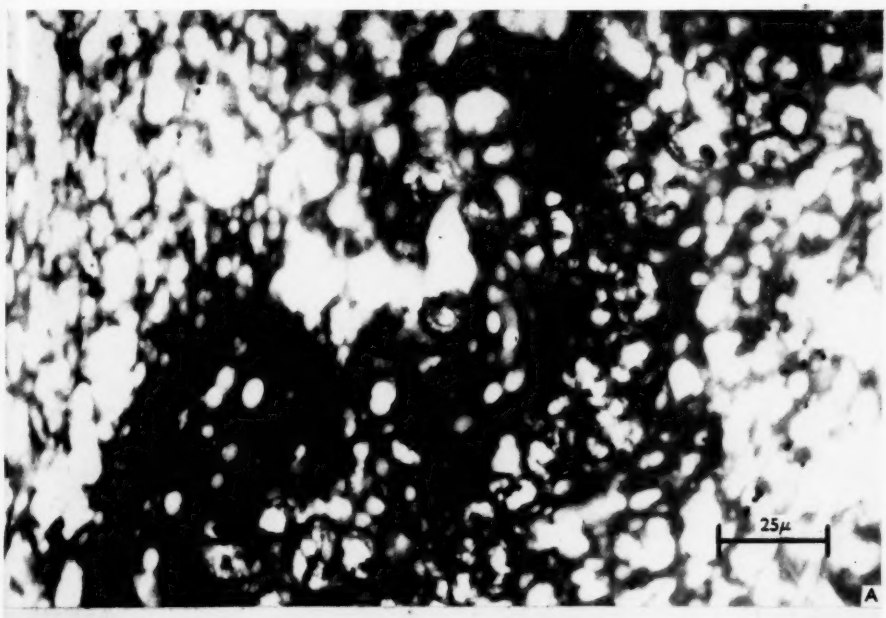
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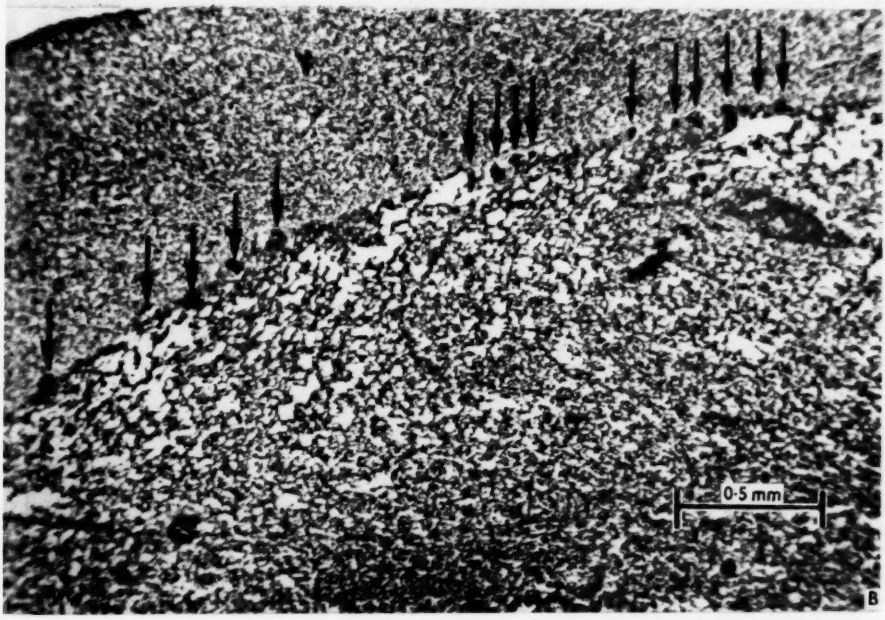
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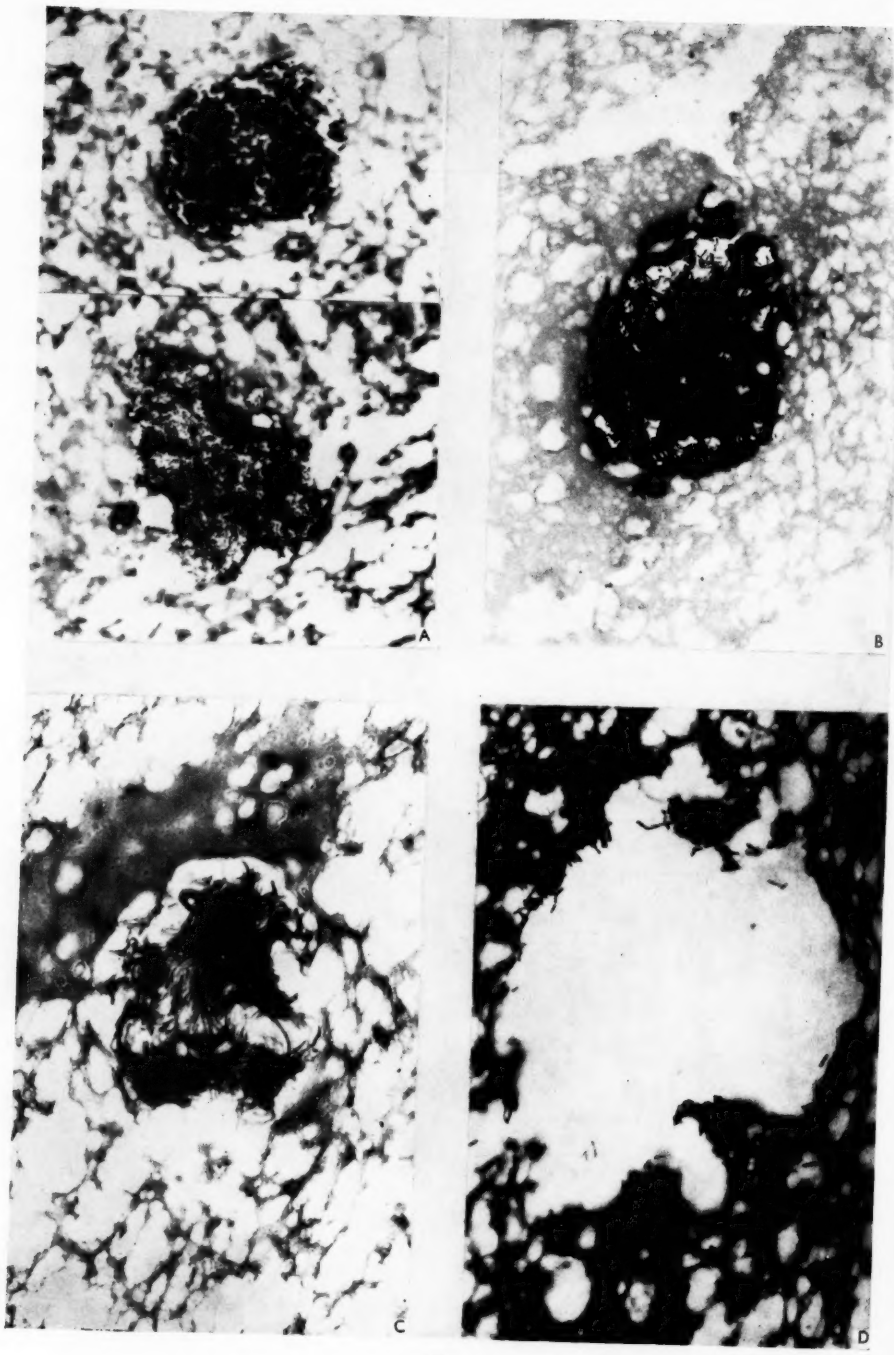
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EXPLANATION OF PLATES

Plate 1

Section of Cheddar cheese, 8 months old. Cheese fixed in formalin, embedded in wax, cut 6μ thick, defatted, then stained by Gram's method. Fine lines are curd junctions. Small, dark areas are, generally, bacterial colonies (see Fig. 1). Single, darkish area at top centre is a 'casein mass' (see text).

Plate 2

Section of Cheddar cheese, 17 months old, prepared as for Pl. 1. Besides curd junctions, 'casein masses', and bacterial colonies, three milled curd junctions (arrowed) are plainly visible (see text). The break across the upper half of the section is a fracture in the intact cheese.

Plate 3

A. Section of Cheddar cheese, 3 weeks old, prepared as for Pl. 1. Annular structures, described in text, are seen distributed along a curd junction. B. Section of Cheddar cheese, 27 weeks old, showing a group of colonies (arrowed) lying on the one curd junction. Cheese fixed in formalin, cut 6μ thick on freezing microtome, and stained with carbol thionin.

Plate 4

Bacterial colonies in sections of Cheddar cheese, fixed in formalin, cut 6μ thick on freezing microtome, and stained with carbol thionin. A, Upper colony, micrococci. Lower colony, small rods. Cheese 39 weeks old. B, Truncated rods. Cheese about 12 months old. C, Closely interwoven, filamentous rods, forming a typical Medusa-head colony. Cheese 26 weeks old. D, Truncated rods lining hole in curd junction. A typical 'angled rod' is clearly visible at the top of the hole. Cheese 46 weeks old.

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With 2 plates

Microscopic observation of the effect of some reagents on the physical state of casein

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SUMMARY. Observations were made by anoptral phase-contrast microscopy of the effect of various reagents on the state of dispersion of casein micelles in skim-milk and in lactic acid and rennet coagula. Among the hydrogen-bond-breaking reagents, urea at higher (4M) concentration dispersed the casein micelles in skim-milk and in acid and rennet coagula. Sodium salicylate was effective at low (M) concentration. Guanidine hydrochloride at low concentration caused aggregation of the micelles in skim-milk but at high concentration caused dispersion, while at both concentrations it dispersed acid and rennet coagula. Lithium iodide and potassium thiocyanate flocculated the casein in skim-milk, but dispersed it in both types of coagula. Flocculation in skim-milk by these reagents was followed by slow coalescence of the casein.

At substantially lower molar concentrations, sodium dodecyl sulphate, an anionic detergent, which attacks either the hydrophobic bonds or the salt linkages in proteins, dispersed micelles and both types of coagula. Sodium thioglycollate, a disulphide-bond-breaking reagent, had no visible effect on the casein.

In rennet coagulum in which the calcium bridges have been destroyed by the sequestering action of EDTA or in lactic acid coagulum, urea at low concentrations caused a quick and almost complete coalescence of casein. Fibres were formed in the rennet coagulum when the coalesced material, which presumably consists of randomly coiled chains of unfolded casein macromolecules, was subjected to stretching or flowing. Similar chemical and physical conditions would appear to prevail when fibre formation takes place in cheddaring cheese curd.

A casein micelle in milk contains a large number of casein macromolecules. The way in which they are spacially arranged and linked together within the micelle and on its surface plays a decisive role in determining the behaviour of casein during various treatments to which milk may be subjected, where the aim may be to retain the original micellar form, to disperse the micelles, or to aggregate them into a coagulum. Similarly, the distribution of casein molecules in the lactic acid and rennet coagulum affects the properties of curd, e.g. during the cheese-making process. In all these cases there are involved intramolecular and intermolecular interactions between the parts of the polypeptide chains, i.e. peptide linkages, between the side chains of different character, as well as interactions between the side chains and the

aqueous environment. The relationships are further complicated by the existence of the several different kinds of casein.

There exists a variety of bonds between the chains, i.e. hydrogen bonds, calcium bridges, salt linkages, hydrophobic bonds, disulphide cross-linkages, etc., which may be broken or weakened by a number of reagents, generally belonging to the denaturing substances. The rupture of bonds in turn leads to rearrangements of the casein molecules (conformation changes), which are followed by changes in the physical state and form of casein. Since a relatively large proportion of the casein micelles in milk are visible in the anoptral contrast microscope, these changes may be readily observed.

EXPERIMENTAL

Skim-milk, lactic acid and rennet coagula, together with the reagents taken by means of a platinum loop, were mixed on a slide, covered with a cover-glass and observed under the anoptral contrast microscope (King, 1959) at a magnification of about $\times 1000$ (anoptral contrast objective 45:1; pair of Kellner eyepieces, $15\times$; binocular body, $1{\cdot}5\times$). The concentrations and pH values refer to the system milk (or clot) plus reagent. The observations were made at room temperature.

The effect of the following reagents was investigated: (1) hydrogen-bond-breaking reagents—urea, guanidine hydrochloride, lithium chloride, lithium iodide, potassium thiocyanate, sodium salicylate; (2) a calcium sequestering reagent—disodium ethylenediaminetetra-acetate (EDTA); (3) an anionic detergent—sodium dodecyl sulphate (which presumably breaks the hydrophobic bonds or salt linkages); (4) an organic solvent—dioxan (which is assumed to break the hydrophobic bonds); and (5) a disulphide-bond-breaking reagent—sodium thioglycollate.

RESULTS

The results of microscopic observations are summarized in Table 1.

(*a*) *Skim-milk*

Untreated skim-milk (pH 6·7) showed a very large number of minute ($< 0{\cdot}2$–$0{\cdot}8\,\mu$ in diameter) spherical casein micelles in vivid Brownian movement.

The bond-breaking reagents either dispersed the casein micelles into particles almost invisible in the anoptral contrast microscope or aggregated them into flocks, with eventual coalescence of the micelles. Various hydrogen-bond-breaking substances behaved differently, and with each substance the effect depended upon the concentration. The dispersing action was exerted by urea and guanidine hydrochloride at higher concentration and sodium salicylate at lower concentration, whereas guanidine hydrochloride at lower concentration, lithium iodide and potassium thiocyanate caused flocculation of the micelles. It is noticeable that the flocks obtained showed a tendency towards the formation of short nodulated fibres, similar to those in the rennet clot from skim-milk diluted 1:10 with 0·1 % calcium chloride (King, 1959). After standing of the specimens for a couple of hours the aggregated micelles coalesced into patches of uniform, structure-less material, in which there occurred numerous droplets, apparently consisting of the serum entrapped in the interstices of the flocks. The edges of such patches were smooth, being different from the granulated

and serrated outlines of the flocks. The formation of fibres was not pronounced. Of other reagents, sodium dodecyl sulphate and EDTA + urea caused dispersion, whereas EDTA alone and dioxan caused abundant flocculation. Sodium thioglycollate was without effect.

(*b*) *Lactic acid coagulum*

The casein in skim-milk was coagulated by dropwise addition of 0·9 M-lactic acid until a pH of 4·8 was reached. The coagulum appeared under the microscope as large dense granular flocks, with no evidence of fibre formation.

With rising concentration of urea an increasing degree of dispersion into single micelles took place. In 4 M-urea the dispersion into invisible particles was almost complete. The micelles in the undispersed part of the coagulum quickly coalesced, entrapping droplets of serum. Occasional currents in the specimen caused flow of the semi-liquid coalesced material and formation of a fibrous pattern in the direction of flow. The concentration needed for complete dispersion of casein was appreciably lower with guanidine hydrochloride and sodium salicylate and still lower with sodium dodecyl sulphate. EDTA alone and in conjunction with urea caused no significant dispersion of the coagulum, which retained its dense granular character. Sodium thioglycollate was without effect, whereas dioxan rendered the coagulum slightly more fluffy.

(*c*) *Rennet coagulum*

The skim-milk was rennetted at 30 °C, the clotting time being 25 min. The pH of the clot was 6·7. As examined under the microscope the clot consisted of densely aggregated casein micelles (Pl. 1, *a*). There was no evidence of fibre formation.

As with lactic acid coagulum, more casein was dispersed into single micelles with increasing concentration of urea and in 4 M-urea the dispersion into very faint particles was almost complete. However, the undispersed clot was of granular character and no coalescence of casein was evident. The complete dispersion was reached at lower concentrations of guanidine hydrochloride and sodium salicylate and at still lower concentration with sodium dodecyl sulphate. Lithium iodide and potassium thiocyanate at higher concentration exhibited partial dispersion and coalescence as well as fibre formation in the undispersed clot.

EDTA in 0·1 M-solution apparently did not exert a dispersing action. However, a treatment with 2 M-urea in addition, apart from a partial dispersion of the clot, caused a nearly complete coalescence of casein. The coalesced material contained numerous droplets, which apparently consisted of the aqueous medium entrapped in the interstices of the originally granular clot (Pl. 1, *b*). In some larger drops some casein micelles in Brownian movement were still present. The semi-liquid coalesced material showed very pronounced ability to form fibres (Pl. 1, *c*), which were drawn out from the mass (Pl. 1, *d*). When the mass was caused to flow by shifting the coverglass in one direction, the formation of fibres became still more spectacular, and in some cases fibres as long as 300 μ could be observed. Sometimes strands of adjoined fibres were present. The coarser fibres on further drawing-out became very thin, about 0·1 μ thick (Pl. 2, *a*), and they ruptured, the broken ends retracting to rounded

Table 1. *The effect of various reagents on the dispersion state of casein, as observed under the anoptral contrast microscope*

Reagent	Concentration	Skim-milk		Lactic acid coagulum		Rennet coagulum	
		pH	Effect on dispersion state of casein	pH	Effect on dispersion state of casein	pH	Effect on dispersion state of casein
Urea	M	7·1	No effect	5·1	Slight dispersion. Undispersed coagulum partly coalesced	6·7	Partial dispersion. Undispersed coagulum granular
	2M	—	—	5·2	Partial dispersion. Undispersed coagulum coalesced. Fibre formation	—	—
	3M	7·4	Almost complete dispersion of the micelles	5·2	Partial dispersion. Undispersed coagulum coalesced. Fibre formation	7·4	Partial dispersion. Undispersed coagulum granular
	4M	7·4	Almost complete dispersion of the micelles	6·4	Almost complete dispersion	7·4	Almost complete dispersion
Guanidine hydrochloride	M	7·0	Abundant flocculation. In about 2 h coalescence of the flocks	5·1	Almost complete dispersion	—	—
	2M	6·8	Almost complete dispersion of the micelles	—	—	6·7	Complete dispersion
	3M	6·8	Almost complete dispersion of the micelles	—	—	6·8	Complete dispersion
Lithium chloride	M	—	No effect	—	—	—	—
	3M	—	No effect	—	—	—	—
	4M	—	—	4·2	No significant dispersion	6·0	Slight dispersion
Lithium iodide	M	—	No effect	—	—	—	—
	3M	6·7	Flocculation. In ca. 2 h coalescence of the flocks	—	—	—	—
	4M	—	—	4·2	Partial dispersion	6·4	Partial dispersion. Coalescence and fibre formation in undispersed coagulum

Table 1 (*cont.*)

Potassium thiocyanate	M	7·2	Flocculation. In ca. 2 h coalescence of the flocks	—	—	—	—
	2M	—	—	6·4	Almost complete dispersion	—	—
	3M	7·4	Abundant flocculation. In ca. 2 h coalescence of the flocks	—	—	—	—
	4M	—	—	—	—	7·8	Partial dispersion. Coalescence and fibre formation in undispersed coagulum
Sodium salicylate	M	7·8	Almost complete dispersion of the micelles	5·2	Almost complete dispersion	—	—
	1·5M	8·3	Almost complete dispersion of the micelles	—	—	7·1	Almost complete dispersion
EDTA	0·1M	5·2	Abundant flocculation	4·2	No significant dispersion. Coagulum granular	5·0	No dispersion
EDTA + urea	0·1M + 2M	5·2	Almost complete dispersion of the micelles	4·7	No significant dispersion. Coagulum granular	5·1	Partial dispersion. Undispersed coagulum coalesced. Fibre formation
Sodium dodecyl sulphate	0·1M	7·4	Almost complete dispersion of the micelles	5·2	Dispersion	8·0	Almost complete dispersion (slow)
Dioxan	50%	5·2	Abundant flocculation	4·7	Slight loosening of coagulum	5·6	No effect
Sodium thioglycollate	0·5%	7·2	No effect	5·1	No effect	7·2	No effect

'stumps' (Pl. 2, *b*). Macroscopically, the EDTA + urea-treated clot appeared as a sticky and elastic lump.

The other calcium-binding reagents, potassium oxalate and sodium citrate, did not exhibit this phenomenon. The former in M-solution (pH 8·4) turned the dense clot into fluffy granular flocks, which slowly disintegrated into single micelles. The flocks contained numerous tiny crystals (0·5–2 μ) of calcium oxalate, which gradually grew larger. Addition of 2M-urea did not change this picture, which was also, except for the formation of crystals, obtained with M-sodium citrate (pH 8·4).

Dioxan and sodium thioglycollate were without effect.

DISCUSSION

The microscopic observations show a variety in the form of interactions between the casein micelles or coagula and different bond-breaking substances. With each of the reagents there may occur differences in the behaviour at different concentrations. A reagent can act as a flocculant in skim-milk, but as a dispersant in the coagula.

The much higher dispersing ability of sodium dodecyl sulphate, as compared with that of urea, is in agreement with the generally accepted view that the anionic detergents, on a molar basis, are very potent denaturants (cf. Waugh, 1954). Kauzmann (1956, 1959) assumed that detergents and organic solvents contribute towards the rupture of the tertiary structure in proteins by weakening the hydrophobic bonds which stabilize this folded configuration, and which are much more important than salt linkages. Lundgren (1949), on the other hand, was of the opinion that the anionic detergent attacks the salt linkages, becoming bound to the basic groups of the protein and assisting to wedge apart the polypeptide chains. Hence the hydrophobic bonds or salt linkages, as well as the hydrogen and calcium bonds, the role of which was stressed by Beeby & Kumetat (1959), may be involved in the stabilization of the structure of the casein micelle. Waugh (1958) explained the remarkable stability of the calcium-caseinate micelles in milk by a primary interaction involving hydrogen bonds as well as the abundant non-polar side chains, followed by the formation of calcium–organic phosphate cross-linkages.

Kauzmann (1959) suggested that aggregation in proteins may be caused by intermolecular hydrophobic bonding between small patches of non-polar side chains on the surfaces of different protein molecules, provided the patches have similar dimensions and are able to come into contact. The occurrence of short nodulated fibres in some kinds of flocks, as reported above, may also indicate the possibility of aggregation by similar intermolecular bonding, in this case between partly or entirely unfolded polypeptide chains of casein.

The behaviour of both types of coagula towards the action of urea and of EDTA + urea was of interest. In the lactic acid coagulum urea caused coalescence of casein to a fibre-producing mass, whereas the rennet clot retained its granular character. The combined action of EDTA and urea had no significant effect on the lactic acid coagulum; with the rennet clot, however, an excessive coalescence to a sticky, elastic mass with pronounced ability to form fibres took place. The formation of fibres was highly accentuated by forcing the mass to flow in one direction. Possibly this particular state of casein is of importance in the fusion of the curd granules, with its

accompanying formation of fibres, during the cheddaring process. Here, the developing lactic acid would be responsible for removal of the calcium, and, together with the raised temperature, for the breaking of some hydrogen bonds. As has been reported previously (Czulak & Hammond, 1956; King & Czulak, 1958), during cheddaring sufficient flow of the curd takes place, and the pH is sufficiently low (Czulak, 1959) to give fibre formation.

It thus appears that the quick coalescence of casein to a mass which on stretching readily forms fibres involves removal of calcium as well as cleavage of hydrogen bonds. The disbanded macromolecules are unfolded and form a mass of entangled randomly coiled chains. On stretching or flowing of the mass the chains are stretched too, and on adlineation they form fibres. The action of urea alone on the rennet clot did not disturb its granular pattern and no unfolding of macromolecules took place, evidently because of hindrance by the calcium bridges. That fibre formation takes place only over a limited pH range is, however, suggested by its absence in lactic acid coagulum treated with EDTA and urea at pH 4·7.

It is of interest that the coalesced mass, the semi-liquid state of which is apparently due to the water acting as dispersion medium for the intertwined polypeptide chains, contains another part of water as spherical droplets, thus closely resembling the picture of a water-in-oil emulsion. Presumably the numerous 'unmasked' hydrophobic side chains provide the necessary non-polar conditions for such dispersion.

The slow coalescence, as occurred in skim-milk with guanidine hydrochloride, lithium iodide and potassium thiocyanate, however, did not require a calcium-binding reagent. The formation of fibres under these conditions was much less conspicuous than occurred with quick coalescence.

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EXPLANATION OF PLATES

PLATE 1

a. Rennet clot.

b. Coalesced casein containing numerous serum droplets in the rennet clot treated with 0·1 M-EDTA + 2 M-urea.

c. Fibres in coalesced casein from the rennet clot treated as for *b*.

d. Drawing-out of fibres from the coalesced casein (rennet clot treated as for *b*).

PLATE 2

a. Drawing-out of coarser fibres into very thin ones (rennet clot treated with 0·1 M-EDTA + 2 M-urea).

b. Retracting of the broken ends of ruptured fibres (rennet clot treated as for *a*).

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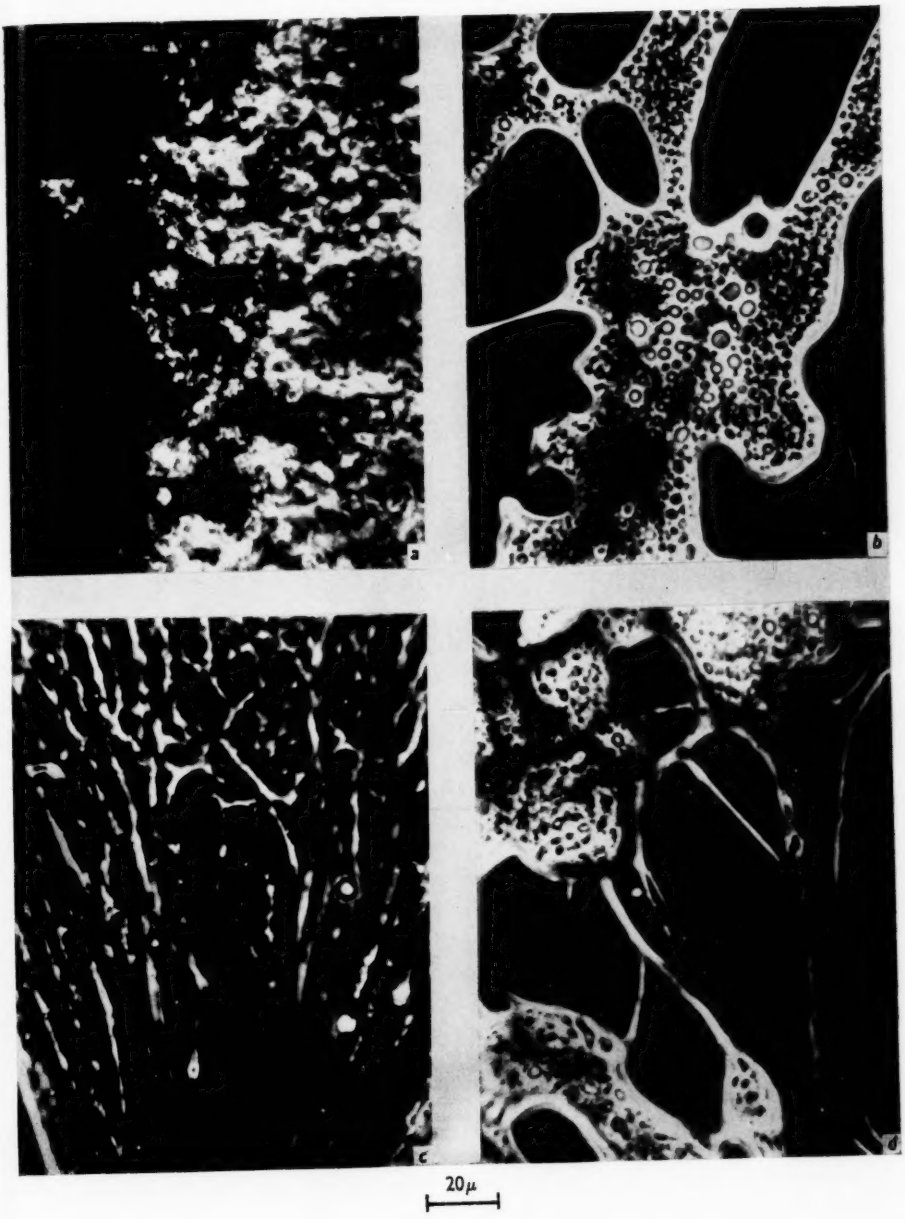

N. KING

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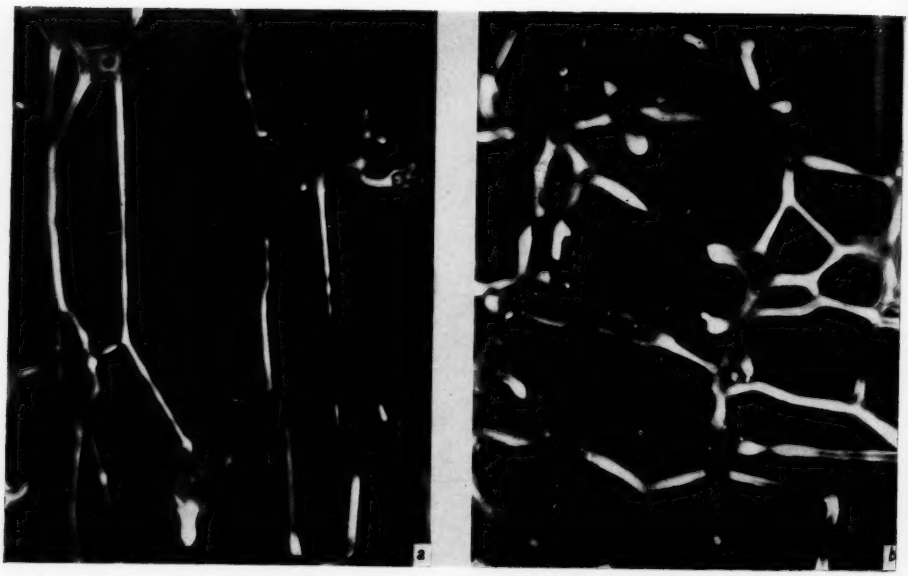
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Genetical aspects of maximum rate of flow during milking

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Summary. Rates of flow of milk during milking have been observed on twin and single-born dairy cows with the aid of a device for continuous recording of weight of milk extracted. Each cow has been characterized by a maximum rate arrived at by averaging morning and evening observations on 2 days. Only this maximum rate has been studied.

Repeatability was high (0·8–0·9) whether measured from day to day or from lactation to lactation. Maximum rate of flow was 10–16 % higher in second than in first lactations. The range extended from under 2 lb/min to over 7 lb/min.

Lactation yield of milk varied significantly with rate, rising about 50 gal for an increase of 1 lb/min. This relation is reduced or eliminated by taking into account by partial regression analysis either maximum weekly yield or the increase from the second to the peak week of lactation.

Although the higher yielding cows had larger udder areas bounded by the four teats, the regression of 305-day milk yield on rate was not changed much by taking udder area into account. Cows with short teats milked faster and had higher yields than cows with longer teats.

Differences in yield among the four quarters widened slightly between 1 and 3 months of lactation regardless of rate of flow, except possibly in the three slowest milking cows. In these the differences increased three times as much as in the other cows. The wider the inter-quarter differences in yield the less the proportion of milk obtained at the maximum rate.

Heritability of maximum rate of flow derived from observations on paired single-born cattle and twins was 85 %. Field data would probably yield a value of 60–70 %. Appropriate selection of breeding stock would effectively alter rate of flow, but there is no reason yet to expect yield of milk to change except perhaps by reducing the incidence of very slow milking cows.

The object of this paper is to extend the observations previously made on the relations between rate of milking and the yield of dairy cows. Fast and slow milking cows must have been recognized since cows were first hand-milked. This limited review of the literature, however, will start with Foot (1935) and Korkman (1948). From them and from Beck, Fryer & Roark (1951) references to some earlier work can be obtained. Foot noticed, as have all workers on the subject, that maximum rate of flow is highly repeatable, which is another way of saying that it is not very sensitive to the usual variables in a cowshed. The statement holds for udders, that is, from

teat to teat of the same udder (Andreae, 1958), for different stages of the same lactation, and for different lactations (Beck *et al.* 1951; Dodd, 1953). Repeatability, however, is not perfect. Maximum rate of flow is said to decline as lactation proceeds (Korkman, 1948; Dodd, 1953; Stewart, Schultz & Coker, 1957) and to change with very unequal intervals between milking (Brumby, 1956). Since peak flow must be a function of internal pressure, external negative pressure, and the anatomy of the udder and teats, variation in any of these factors will alter it (see Baxter, Clarke, Dodd & Foot, 1950; Whittlestone, 1955; Dodd & Clough, 1957; Loppnow, 1959).

Dodd & Foot (1953) observed that high-yielding cows tended to be fast milkers. For their material there was a positive regression of 419 lb of milk in a 305-day lactation yield for a change of 1 lb/min in the maximum rate of flow. This led them to contemplate culling slow milkers and breeding from the fast ones with a view to higher yields. Sandvik (1957*a*) and Johansson & Malven (1960) obtained similar results, and observed that the regression of yield on rate was non-significant when daily production at the time of observing the rate was held constant.

Sandvik (1957*b*) also studied udder shape in relation to milking rate but found no relation between them. He did find, however, as did Johansson & Malven (1960) an increase in rate of flow as teat length diminished. Some connexion between teat dimensions and the efficiency of fixed-size teat-cup liners seems quite likely.

Estimates of heritability of rate of flow have been given by Brumby (1956) and Donald (1958). Both used twins and arrived at estimates of the order of 80%. More extensive material being now available revised estimates will be given below. Johansson (1958) reported a heritability of 35% estimated from single observations made in a field study of commercial cows.

EXPERIMENTAL

Material and methods

The cows studied all belonged to the Animal Breeding Research Organisation. Some were in the Blythbank twin herd (Donald, 1953) and some in the Cold Norton herd devoted to cross-breeding. In both herds, most cows had their first calves when 2–2¼ years old and were disposed of after two lactations, but before this there was no culling except for infertility or gross ill health. In both herds also, various breeds and crosses were kept.

Milking was by machine only. For present purposes the mechanical aspects of the milking process can be regarded as uniform within herds. Observations on milk flow were taken by suspending the usual milking unit from a special device made by Mr George Newell. His device produces a continuous record of the weight of milk extracted and is essentially a spring balance recording weight on a revolving drum driven by clockwork. On the surface of the drum is fixed a specially printed sheet of paper with time and weight scales. From it several pieces of information can be read off including the maximum rate of flow. An easy way of doing this is to read the angle which the flow curve makes with the base line. Either the angle or the corrresponding 'pounds per minute' can be taken as the variable. The relationship between the two of course is not quite linear.

At Blythbank, Alfa-Laval milking machines were used at a vacuum of 14·0 in.

Hg, and a pulsation rate of 50 c/min. At Cold Norton, the machines worked at 14·5 in. Hg, and 40 c/min. Milking intervals on both farms were approximately 10 and 14 h.

Except for the uniformity trial to be mentioned, analyses have been based on the means of sets of four observations, two at morning milkings and two at afternoon milkings, taken approximately 1 month after calving. Another set was taken at approximately 2 months after calving, but these sets included a number with only two observations. Many cows provided more than two sets.

To obtain an estimate of the error attached to a particular observation a uniformity trial was carried out on five cows. These were observed twice a day for 10 days during a fortnight. Within cows, the over-all variance was 0·119 (lb/min)^2. When allowance was made for differences between days, and between morning and afternoon milkings, the variance was reduced to 0·069 (lb/min)^2. It is a matter of some interest that significant day-to-day variation occurred under apparently uniform conditions.

For seventy one-egg pairs and seventy-eight two-egg pairs of twins, with more widely spaced sets of observations, the within-cow variances of a single routine test (date and time of day ignored) were 0·222 and 0·174 (lb/min)^2 respectively, that is, somewhat larger than the value of 0·119 found in the uniformity trial.

RESULTS

Means and repeatabilities

Mean rates of flow derived from the sets of four recordings for each cow in the twin herd at Blythbank are set out in Table 1.

Some of these cattle were twins and some singles. Nearly all were purchased when a few days old without reference to the performance of their parents, and may be

Table 1 *Mean maximum rates of flow (lb/min) by breed and lactation number (Blythbank herd)*

	First lactation		Second lactation	
Breed	No.	Rate	No.	Rate
Friesian	40	5·1	24	6·0
Ayrshire	106	4·3	51	4·1
Shorthorn	18	4·7	12	5·3
Miscellaneous	48	4·3	28	4·6

regarded as random samples of the several breeds involved. Friesians reached a greater average maximum rate than the other cattle both as heifers and as second calvers. If, however, correction is made for differences in milk yield, the differences in rate of flow between breeds disappear. Although the Ayrshires in the Blythbank herd failed to show an increased rate in second lactations the other groups in this herd and those at Cold Norton did. This is shown in Table 2 which is limited to cows with two lactations.

In the second lactations the average rate of flow was 10–16 % higher than in first lactations. It rose in fact proportionately as much as the 305-day milk yield, but not

as much as the maximum weekly yield (30–36 %). There was a decline of 7 % in the mean lactation length (Table 2). The maximum milking rate varied from under 2 lb/min to over 7 lb/min (Table 8). A cow milking at 2 lb/min obviously takes at least 10 min at her full rate to deliver 2 gal of milk. Such cows have been observed to maintain a steady rate for 10 min.

Mean maximum rates of flow from first and second lactations are strongly correlated. For the twin herd, the data for thirty-seven pairs of cows have been tested in two lactations with the results shown in Table 3. Numbers are small but the subgroups agree in showing that the correlation coefficient to be expected for unrelated animals would be 0·8–0·9. Mean maximum rate of flow from first and second lactations of the same animal are strongly correlated as shown by the 'total' coefficients (in line 2 of Table 3) which are about +0·8. Differences among pairs in first

Table 2. *Ratios of yield characteristics of second to first lactations*

Farm	Breed	No. of cows	305-day yield	Max. wk. yield	Rate of flow	Lact. length
B.B.	Friesian	17	1·12	1·30	1·11	0·98
B.B	Ayrshire	46	1·09	1·30	1·05	0·91
B.B.	Other	27	1·03	1·30	1·17	0·93
B.B.	All	90	1·08	1·30	1·10	0·93
C.N.	All	216	1·16	1·36	1·16*	0·93

B.B. = Blythbank; C.N. = Cold Norton.
* Based on 35 cows.

Table 3. *Correlation of maximum rates of flow in first and second lactations (not within breeds except within pairs)*

	MZ	*DZ*	*HS+U*
No. of pairs	10	16	11
Total	0·78	0·84	0·88
Between pairs	0·81	0·86	0·92
Within pairs	0·27	0·78	0·86

MZ = Monozygotic twins; *DZ* = dizygotic twins; *HS+U* = half-sister pairs and pairs of unrelated animals.

lactations are also correlated strongly with subsequent differences among pairs in second lactations (line 3 of Table 3). Both of these sets of coefficients are compatible with highly persistent effects of heredity or of environment or of any combination of them. Within pairs, however, the coefficients are not uniformly high (line 4). Where genetic variation can be assumed within pairs (i.e. in dizygotic and half sister plus unrelated pairs) the difference between the members of a pair persists strongly from first to second lactation; but where only environmental variation occurs within pairs (as in monozygotic pairs) any difference between members of a pair in first lactations is only weakly correlated with the difference between them in second lactations. From the correlations in Table 3, therefore, it is inferred that heredity is largely responsible for the observed variation among cows in rate of flow.

Rate of flow and milk yield

Results from both Blythbank and Cold Norton agree with those of Clough & Dodd (1957) and Sandvik (1957*a*) in showing a significant association between rate of flow and lactation yield. High-yielding cows tend to milk fast. In terms of a regression, there has been a change of about 53 gal of milk for each change of 1 lb in the rate of flow per minute (Table 4). Cows in their second lactation at Cold Norton, however, showed only a 27 gal change. Because the change in yield was small for a relatively large change in rate, only 11 % or less of the variation in yield is eliminated by holding the rate constant.

Following Sandvik (1957*a*), statistical allowance was made for milk yield at the time of observing rate of flow. For the present purpose, partial regressions of 305-day yield on rate have been calculated with lactation maximum weekly yield held constant, and appear in Table 5. Although the partial regression is almost significant for the Blythbank herd, it is effectively zero for the Cold Norton herd.

Table 4. *Regression of* 305*-day milk yield* (*Y*, *lb*) *on rate of flow* (*A*, *lb/min*)

Farm	Lact. no	No. of cows	b_{YA}	S.E.	% of variance in *Y* removed by *A*
B.B.	1	178	537	124	10
C.N.	1	87	528	182	11
	2	83	270	175	3
Clough & Dodd (1957)	—	352	381	56	—
Sandvik (1957*a*)	—	111	439	—	—

Table 5. *Regression of* 305*-day yield of milk* (*Y*, *lb*) *on rate of flow* (*A*, *lb/min*) *and maximum weekly yield* (*M*, *lb*)

	$b_{YA.M}$	S.E.	% of variance in *Y*	
			Removed by *A*	Removed by *M* and *A*
B.B. 178 cows within type of pair (not within breed)	157	85	10	61
C.N. lact. 1, 87 cows within breeds	5	121	11	67
C.N. lact. 2, 83 cows within breeds	−1	119	3	59
Clough & Dodd (1957) 135 cows	326	81	For cows giving about 1½ gal/milking	
Sandvik (1957*a*) 52 cows	117	N.S.		

B.B. = Blythbank herd; C.N. = Cold Norton herd. N.S. = not stated.

Since lactation yield is so dependent on peak production it is not altogether surprising that this partial regression should be zero or nearly so. It follows, however, that the maximum weekly yield was itself dependent on rate of flow, and that if rate had any important effect on lactation yield, this effect was achieved very early in lactation. It turns out that for the 178 cows at Blythbank mentioned in Table 4, the regression of maximum weekly yield on rate of flow was $10{\cdot}6 \pm 2{\cdot}6$ lb per lb/min.

For the eighty-seven Cold Norton cows it was $13{\cdot}3 \pm 3{\cdot}7$ lb, a highly significant result but accounting for only 15 % of the variation in maximum weekly yield.

If slow milking deterred cows from realizing their potential yields, it might do so by reducing the increase expected during the first few weeks of lactation. Within types of pair (but not within pairs or breeds) at Blythbank the regression of the increase in yield from the second to the peak week on rate of flow was significant but trifling, $5{\cdot}2 \pm 1{\cdot}6$ lb per lb per min. Only 6 % of the sum of squares for the increase could be accounted for by differences between cows in rate of flow.

Rate of flow and udder characters

Most udder measurements appear to be without interest in this connexion in the sense that they showed no significant medial correlations in the present material with either milk yield or maximum rate of flow. Udder area, however, showed a medial correlation with lactation yield within pairs of singles and of twins that approached significance with a coefficient of $+0{\cdot}21$. This character therefore and teat length (following Sandvik's observations) are the only ones to be considered further here. Udder area is the area defined by the points of the four teats and teat length is the shrunk length obtained when teats are lightly touched. Both measurements were taken about 6 weeks after calving and just before milking.

Table 6. *Partial regression* (b) *of lactation yield and daily yield at the time of observation on udder area* (U, cm^2) *and rate of flow* (A, *lb/min*) *for cows at Blythbank* (*B.B.*) *and Cold Norton* (*C.N.*)

Yield, lb.	Farm	D.F.	$b_{YA.U}$	S.E.	$b_{YU.A}$	S.E.
305-day	B.B.	158	446	126	5·35	1·36
	C.N. (Lact. 1)	68	460	178	4·92	1·54
	C.N. (Lact. 2)	66	265	171	1·88	1·55
Daily	B.B.	158	1·31	0·37	0·016	0·004

According to the figure in Table 6, there is a significant increase in 305-day yield of milk as rate of flow increases even when allowance is made for udder area. The partial regression coefficients are in fact much like the simple regression coefficients in Table 4, which implies that udder area makes little difference to the relationship. Nevertheless, there is, as might be expected, a tendency for high-yielding cows to have large udder areas. For each square centimetre of udder surface bounded by the teats, lactation yield rose by about 5 lb. The partial regression of yield on udder area independent of rate of flow, however, accounts for only 9 % of the sum of squares for the 305-day yield. Table 6 also shows that when daily yield of milk at the time the rate of flow is recorded replaces 305-day yield in the calculations, similar conclusions about the relationships of the three characters may be drawn.

Sandvik (1957*b*) has made the interesting observation that maximum rate of flow tends to be higher in cows with short teats than in those with long teats. To see whether this applied to the Blythbank cattle, they were classified by breed and teat length. Some of the characteristics of the resulting classes appear in Table 7. The figures suggest that the cows with teats of a shrunk length under 4 cm not only yield better than those with longer teats regardless of breed, but also tend to milk

faster. On previous evidence, of course, it is to be expected that those two traits would vary in accord with each other. To all intents and purposes these cattle were unculled and offer useful confirmation of Sandvik's assertion. The Friesians, it will be noticed, included more cows with comparatively high maximum rates of flow than did the other cattle available.

Another udder character that has been examined is a measure of symmetry called the least quarter ratio, calculated from the individual yields of the four quarters near the time of observing the rate of flow, that is at 1 month and 3 months after calving. The ratio is four times the production of the lowest yielding quarter over the sum of the yields of the four quarters. Behind the choice of this character lies the idea that in a slow-milking cow the most productive quarters might be the ones

Table 7. *Differences in yield characteristics of heifers in the Blythbank herd classed by teat length*

Breed	Teat length, cm	No.	305-day milk yield, gal	Max. weekly yield, lb	Rate of flow, lb/min	Lactation length, weeks
Friesian	Up to 3·9	9	772	242	5·7	40
	4·0–4·9	21	640	249	4·7	37
	5·0 and over	7	657	237	4·1	43
Ayrshire	Up to 3·9	36	689	259	4·4	39
	4·0–4·9	57	622	241	4·1	37
	5·0 and over	6	530	220	4·4	31
Misc.	Up to 3·9	18	612	244	4·2	37
	4·0–4·9	32	583	222	4·1	38
	5·0 and over	11	598	215	3·9	35

to suffer most if the yield were affected by rate of milking. If this were so, the ratio would increase as time went on, and the best quarter became more like the worst. The lowest yielding quarter at 1 month and 3 months after calving was not necessarily the same quarter, but it was always a front quarter. Had the 'lightest' quarter been defined solely by the first recording, a small increase in the ratio would have been expected owing to a re-distribution of random variation in quarter yields. For the purpose of examining this possibility, cows were classed by rate of flow into seven categories, and the corresponding average least quarter ratios worked out. They are shown in Table 8, as well as those for a special category of cows showing very low ratios following mastitis.

Far from increasing, the ratio tended if anything to diminish between the ends of the first and third months of lactation (cf. Crossman, Dodd, Lee & Neave, 1950). Furthermore, the only suggestion that slow and fast milkers can be distinguished either by the ratio or by any alteration in it is offered by the three very slow-milking cows. In these the ratio decreased three times as much as in the remainder.

The sixteen cows with low ratios failed to show any change of ratio. The lowest yielding quarters of these cows (which were not subject to recording until clinically normal) apparently maintained their relative position among the four quarters over the two months concerned.

It is a common experience that the fore quarters appear to milk out more quickly than the rear quarters. There might accordingly be a positive correlation between

the least quarter ratio as defined above and the fraction (known as the part-yield) of the total yield of milk that is in the bucket when the graphical record shows the maximum giving way to a lower flow. In other words, the nearer equal the quarters are in production, the larger the part yield. The correlation coefficients using part yields based on all flow recordings obtained were +0·28 one month after calving and +0·35 three months after calving (140 D.F.). Although not very strong, the correlation has the expected sign. A partial correlation, with total yield constant, might well be larger. This has not been calculated, but the expectation is heightened by the evidence from twenty pairs of monozygotic twins. Their part yields have been correlated not only with the least quarter ratio but also with the fore quarters ratio, that is, the proportion of total yield due to both fore quarters. Table 9 contains the results.

Within healthy one-egg twin pairs there is little, perhaps no, correlation of part yield with either of the ratios, but between pairs there is a substantial correlation. This result is interpreted as meaning that there are heritable differences in the balance

Table 8. *Relation between least quarter ratio and maximum rate of flow at 1 month and 3 months after calving*

$$\text{Least quarter ratio} = \frac{(4 \times \text{production of lowest yielding quarter})}{\text{Sum of yields of the 4 quarters}}.$$

Rate of flow, lb/min			Least quarter ratio	
Range	Mean	No.	1 month	3 months
Up to 1·9	1·77	3	0·79	0·70
2·0–2·9	2·55	20	0·79	0·75
3·0–3·9	3·48	38	0·79	0·75
4·0–4·9	4·39	37	0·81	0·79
5·0–5·9	5·46	30	0·81	0·77
6·0–6·9	6·45	8	0·79	0·77
7·0–7·9	7·55	6	0·81	0·76
All		142	0·80	0·77
Cows with low ratios (< 0·6 at 1 month)		16	0·33	0·34

Table 9. *Correlation of part yields with fore-quarters and least quarter ratios: 20 MZ pairs*

	D.F.	Part yield and least quarter ratio	Part yield and fore quarters ratio
Between pairs	18	+0·68	+0·57
Within pairs	19	+0·20	+0·01

of front and rear quarters as already reported by Johansson & Korkman (1952) and Johansson (1957); and the variation in balance affects part yield in such a way that the nearer equal the yields of front and rear quarters, the greater the part yield and the smaller the amount of milk to be extracted at reduced rates of flow. To confirm this there are the three averages. For the forty cows the average part yield was 0·74. The average value for the least quarter ratio was 0·76 which is good agreement provided that the duration of maximum rate of flow for a quarter is linearly proportional to its content. Fore quarters ratios on average were 0·58—also good agreement since the sum of the two quarters will be greater than twice the lesser.

Teat shape has been related to rate of flow but, as Sandvik (1957*b*) also found, it seems to make no appreciable difference to the rate whether teats are parallel-sided or slightly narrowed towards the tip. Only for these two classes of teat were there sufficient cows to permit a satisfactory comparison.

Heritability of rate of flow

Few estimates of heritability have been found in the literature, but some writers give the impression that they expect maximum rate of flow to be strongly inherited. From thirteen sets of monozygotic twins, Brumby (1956) estimated heritability (h^2) as 72·4%, and from fourteen sets of dizygotic twins 86·2%. With rather more of the same kind of material, Donald (1958) later found a value of 90%.

There are now available not only more twins, but contemporary pairs of paternal half-sisters and pairs of unrelated animals, all raised as twins under a uniform system of management. By keeping these single-born animals under the same conditions as the twins, it is possible to look for harmony in the series of pairs of varying degrees of relationship without the complication that arises if animals reared as twins and animals reared as singles are not equivalent in respect of the amount or distribution of environmental variation.

Table 10. *Intra-class correlations (within breeds, within pairs) for maximum rate of flow within pairs of twins and singles reared as twins*

	MZ		*DZ*		*HS+U*	
	No. of pairs	*r*	No. of pairs	*r*	No. of pairs	*r*
Lact. 1	25	0·656	29	0·591	34	0·157
Lact. 2	14	0·983	21	0·371	14	−0·033
Combined	39	0·857	50	0·491	48	0·104

(*MZ*, monozygotic; *DZ*, dizygotic; *HS*, half-sisters; *U*, unrelated animals.)

From the variances within and between pairs of the four types, intra-class correlations measuring the resemblance of pair mates have been estimated and are given in Table 10. Pairs of half-sisters have been combined with pairs of unrelated animals and treated as unrelated. The effect of doing so should be to underestimate a little the amount of genetic variation within unrelated pairs. Data from the first and second lactations are shown separately and combined, although some pairs occur in both.

In broad terms, strong intra-class correlations for all groups would imply a strong correlation of environments for pair mates. Strong correlations for monozygotic twins, intermediate ones for dizygotic twins and weak ones for the unrelated pairs imply that environments are not, or only weakly, correlated for pair mates, and that heredity is responsible for most of the variation. This is what will be found in Table 10. A saving clause must be inserted here to the effect that pre-natal environment common to twins of a pair could conceivably cause differences among pairs that are not distinguishable in this material from differences due to the heredity of the twins. If this were happening to any great extent, however, the dizygotic twins should behave like monozygotic twins instead of adopting a position midway between monozygotic

and unrelated pairs. These conclusions apply, strictly speaking, only within the limits of environmental variation, as it affects rate of flow, on one farm.

From the intra-pair variances of the several types of pair, it is possible to derive estimates of heritability (King & Donald, 1955). These depend on the assumptions that variance within monozygotic pairs is wholly environmental (e^2), that within dizygotic pairs it contains additionally half the genetic variance extant in the population from which they are drawn ($e^2+\frac{1}{2}g^2$), and that in unrelated pairs it consists of (e^2+g^2). Proceeding from these assumptions, the argument goes on to develop estimates of heritability (h^2) by equating h^2 to $g^2/(e^2+g^2)$. This ratio can be obtained from any two of the three classes of pair in Table 10. The estimates are as follows:

$$\text{Heritability from } (DZ\text{–}MZ) = 85\,\%$$
$$(U\text{–}MZ) = 85\,\%$$

Such good agreement was not to be anticipated. However, it leaves no doubt that cows are easily classified for rate of flow, and could if necessary be effectively selected for this character on the basis of their own performance.

A guess can be made at the level of heritability that would be shown by data obtained, as milk records are, from many herds and sire progenies. Experience shows (Donald, 1958) that an anatomical character of a mature beast will manifest a rather smaller proportion of genetical variation in the field than in an experimental herd. Heritability would probably be of the order of 60–70 % instead of 85 %, if rate of flow were observed with the frequency and accuracy applied to the present twins.

DISCUSSION

Maximum rate of flow is only one, but an important one, of the factors determining total machine time (Beck *et al.* 1951; Dodd & Clough, 1957). It is likely to be the most highly heritable since it depends largely on an anatomical trait—the teat canal. By culling the slowest milking cows, a herd average for maximum rate of flow would rise immediately, and some of this effect would be carried over into the next generation.

There would be a concomitant change in milk yield that could be estimated from the observed regression coefficients, but this would apply only to the surviving cows of the current herd and it would be quite small. It may be suspected that the offspring of the survivors, although milking a little faster, will not in consequence yield any more milk. Until it is known, however, that there is no genetic correlation between yield and rate of flow, no assertions can be made.

The fact that there is a significant regression of yield at all stages of lactation on maximum rate, at least under some circumstances, counsels caution for there is as yet no explanation of this regression. If it eventually becomes clear that slow milking due to narrow teat canal does place a check on the realization of full milk potential, then removing this check, by, for instance, modifying the machines, will raise yields without the help of a positive genetic correlation between yield and rate. Teat canals so narrow as to reduce milking rate below 2 lb/min and extending machine time to over 10 min have occurred several times in the present material. For cows with such teats there must be a risk of incomplete milking—if not through haste by the milker then by failure of the let-down stimulus to last long enough. A few cows like this, however, would not be enough to account for the observed regression.

It should be noted that the three healthy cows that milked at maximum rates under 2 lb/min had an average lactation yield of 337 gal instead of the 500–600 gal predicted by the regression equation. This deviation from linearity may not be merely accidental.

Although some interesting questions about milking arise from these and earlier observations (Dodd & Foot, 1953) it seems unlikely that the recording of field data on rate of flow will do much in the cause of higher yields in herds where stockmanship sees to it that the slow-milking cow has the time she needs. Other circumstances arise, however, in which the husbandry system does not allow for special treatment. If a cow needs 10 min and is given 5, her yield will suffer. Since the slow-milking cow cannot adapt herself to fast milking she will be culled for low yield (Dodd & Foot, 1949). Instead of culling her at the end of a poor lactation, an owner might have tested her rate of flow at the start, and if necessary sold her to someone to whom it was immaterial.

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Fishy flavour in dairy products

III. The volatile compounds associated with fishy flavour in washed cream

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Summary. When washed cream was treated with copper and ascorbic acid and held at 2 °C for 1–5 days a strong metallic, fish-oil flavour developed. Compounds responsible for the flavour were isolated and identified. Gas chromatographic separation of a flavour concentrate on a silicone oil column gave six characteristic flavour fractions which, in order of elution, were described as painty or drying oil, oily, mushroom, metallic, tallowy and cucumber. The flavours of all fractions were predominantly caused by carbonyl compounds and the oily and metallic fractions were the main contributors to the typical fish-oil flavour. The following compounds were characterized: n-pentanal and pent-2-enal in the painty fraction; n-hexanal, n-heptanal and hex-2-enal in the oily fraction; hept-2-enal and a carbonyl compound with a mushroom odour in the mushroom fraction; a carbonyl compound with a metallic odour in the metallic fraction; n-octanal, n-nonanal, oct-2-enal and hepta-2,4-dienal in the tallowy fraction; and non-2-enal in the cucumber fraction.

The conditions responsible for the production of fish-oil flavour in butterfat treated with the antioxidant nordihydroguaiaretic acid (NDGA) and citric acid, and the characterization of the compounds responsible for the flavour have been reported in two earlier papers (Pont, Forss, Dunstone & Gunnis, 1960; Forss, Dunstone & Stark, 1960*a*). The fish-oil flavour was shown to contain two components, an oily and a metallic. The oily flavour component was itself a mixture and contained n-hexanal, n-heptanal and hex-2-enal, while the metallic flavour appeared to be caused by a single powerful tainting carbonyl compound.

During the above investigation a striking resemblance was observed between the fishy flavour under examination and the flavour induced in washed cream by ascorbic acid and copper (Olson & Brown, 1942) or by ascorbic acid alone (Forster & Sommer, 1951; Pont, 1952). Pont reported that this flavour in washed cream, which he described as suggesting mown grass or linseed oil, was quite different from the cardboard flavour of oxidized skim- or whole milk. More recently, workers in the Netherlands (Tarassuk, Koops & Pette, 1959; Koops, Tarassuk & Pette, 1959), in a detailed study, described the flavour induced in washed cream by copper and acid as trainy or fishy. As part of the present investigation butterfat with fish-oil flavour produced by NDGA and citric acid, and butter with fish-oil flavour made from washed cream treated with copper and ascorbic acid, were compared with cultured cream Dutch butter with a

trainy flavour by tasting. It was found that the flavours were very similar but that of the Dutch butter was slightly more painty and less metallic.

There are considerable physical and chemical differences between the washed cream systems and butterfat. Investigations were therefore undertaken to isolate and identify the compounds formed in the washed cream so that they could be compared with those previously isolated from fishy butterfat. These gave incidental information on compounds causing a variety of other flavours in oxidized lipids.

EXPERIMENTAL

(1) *Preparation of fish-oil flavoured cream and butter*

Thirty gal lots of pasteurized milk were heated to 36 °C and separated to give a cream of 48 % butterfat. The cream was mixed with warm water to 30 gal, warmed to 36 °C, and re-separated. This operation was repeated once. The washed cream (40 % butterfat) or in some cases the buttermilk or butter serum obtained after churning it, were treated with 5 ppm. copper as cupric chloride and with 50 ppm. ascorbic acid. The fish-oil flavour developed immediately and was allowed to intensify by holding at 2 °C for 1–5 days.

The most useful source of fish-oil flavoured compounds proved to be butter prepared from treated washed cream. The flavour of this butter, which appeared identical with that of the cream, buttermilk and serum, was described by a laboratory panel as oily, metallic, fish-oil or cod-liver-oil, while Dairy Produce Officers of the Department of Primary Industry described it as fish-oil, cod-liver-oil, sardine-oil and shark-oil. The butter could be stored at −15 °C for 2–3 months with no loss of the fishy flavour, although a slight tallowy flavour was often observed a week or two after churning. In recent work attention has been concentrated upon the metallic component, and butter from unwashed cream treated with copper and ascorbic acid has been found the most useful source.

(2) *Distillation from butter*

Two kg lots of butter were melted slowly, placed in a separating funnel and the butterfat and serum collected. A vacuum distillate of the butter serum was prepared. This was boiled to provide the steam for the distillation of the butterfat by the procedure previously used (Forss *et al.* 1960*a*).

(3) *Characterization of flavour compounds*

The methods used for the isolation and identification of the volatile compounds, mainly gas chromatography, absorption spectrophotometry and characterization of the 2,4-dinitrophenylhydrazones, were similar to those already described (Forss *et al.* 1960*a*). Greater use was made of gas chromatography. Mass spectra were also obtained, most measurements being made in the high-resolution mass spectrometer recently constructed by the Division of Chemical Physics of C.S.I.R.O. (masses could be measured to an accuracy of 1 part in 25000).

The light petroleum extracts were concentrated at low temperature. One to three ml amounts of the concentrate were added to programmed gas chromatographic columns with silicone oil and 'Carbowax 400' as stationary phases. The odour of the

eluted gas was noted. Fractions were condensed and added to bland butterfat for flavour evaluations. The 2,4-dinitrophenylhydrazones of the separated compounds were prepared. These were identified by the properties listed in Table 3 of the earlier paper (Forss *et al.* 1960*a*).

(4) *Effect of various substrates and procedures*

The effect on the gas chromatograms of washing the cream, the effect of added copper and ascorbic acid and the effect of storage of the butter were studied. Comparison was made with corresponding gas chromatograms from the fish-oil flavoured butterfat previously studied.

RESULTS

The steam distillate from fish-oil flavoured butter had a strong cod-liver-oil aroma and, when examined in a spectrophotometer, had a light absorption maximum (λ_{max}) at 222 mμ with a smaller peak at 275 mμ. Examination of the light petroleum extract showed a λ_{max} of 221 mμ and a smaller peak at 267 mμ, probably due to alk-2-enals, and n-alkanals and alka-2,4-dienals respectively.

Separation of the effluent from gas chromatographic columns on a flavour basis gave six fractions which were described in order of elution as painty or drying oil, oily (like that of paraffin oil or engine oil), mushroom, metallic, tallowy and cucumber. When these fractions were added to water, to N-H_2SO_4 and to N-H_2SO_4 containing 2,4-dinitrophenylhydrazine, the odours, when observed either directly or by distillation and addition of the distillate to bland butterfat, were unaffected by the water and H_2SO_4 but disappeared on treatment with 2,4-dinitrophenylhydrazine. The flavours of all the fractions thus appeared to be due to carbonyl compounds.

A silicone oil gas chromatogram showed eleven major peaks with retention times in order of elution corresponding to n-pentanal, pent-2-enal, n-hexanal, hex-2-enal, n-heptanal, hept-2-enal, n-octanal, hepta-2,4-dienal, oct-2-enal, n-nonanal and non-2-enal. Only a trace of heptan-2-one, much less than in the previous investigation on butterfat, was isolated with the n-heptanal. When the compounds corresponding to these eleven peaks were trapped separately and added to bland butterfat, their flavours with the exception of hept-2-enal were similar to, but not always identical with, those obtained from the same aldehydes fractionated from a synthetic mixture on the same column.

The six flavour fractions were found to correspond to the following groups of compounds: the painty fraction contained n-pentanal and pent-2-enal; the oily fraction contained n-hexanal, n-heptanal and hex-2-enal; the mushroom fraction contained as a major odour constituent a carbonyl compound not yet identified, and hept-2-enal; the metallic fraction has not yet been fully resolved; the tallowy fraction contained n-octanal, n-nonanal, oct-2-enal and hepta-2,4-dienal; and the cucumber fraction non-2-enal.

Tests of the flavour resulting from various combinations of the flavour fractions showed that the oily and metallic elements made the most significant contributions to the fish-oil flavour. More detailed observations were made on some of the fractions.

Particular attention was given to the identification of the compound responsible

for the cucumber flavour for the following reasons: Takei & Ono (1939) had shown that nona-2,6-dienal was the chief odour compound in cucumbers; Harrison & Daubert (1953) had isolated deca-2,6-dienal from heat-oxidized methyl linoleate; and alk-2-enals and alka-2,6-dienals are similar both as the free compounds and as 2,4-dinitrophenylhydrazones. Non-2-enal and nona-2,6-dienal were closely compared. The results of this comparison, reported in another paper (Forss, Dunstone & Stark, 1960*b*), showed that the compound in the flavour fraction from washed cream was non-2-enal.

The mushroom and metallic fractions present difficult problems in identification because the two compounds taint very powerfully and are present in very small amounts, and because the preparation of their 2,4-dinitrophenylhydrazones may be complicated by the simultaneous formation of osazones.

Fractionation of the light-petroleum-soluble 2,4-dinitrophenylhydrazone derivatives on Celite-nitromethane liquid chromatographic columns yielded several bands. The first of these appeared to be the significant compound in the mushroom flavour: when the derivative was dissolved in 50 % sulphuric acid and the solution diluted with water the characteristic mushroom odour was obtained. The 2,4-dinitrophenylhydrazone was further purified by passage through an alumina adsorption column. It had the following properties: λ_{max} in ethanol, 368 mμ; λ_{max} in ethanol-sodium hydroxide solution, 455 mμ; movement on methanol-heptane paper chromatogram the same as for n-nonanal derivative; infra-red spectrum in micro-KBr disk showing the presence of an hydroxyl group.

Hept-2-enal was eluted with the mushroom flavour fraction and although it occurred in larger amounts than in the fish-oil flavoured butterfat treated with NDGA and citric acid, made only a minor contribution to the flavour.

The metallic fraction is represented on the chromatogram by a small peak between those corresponding to hept-2-enal and n-octanal and it has been difficult to obtain free from contamination. The observation that butter from unwashed cream treated with copper and ascorbic acid contained relatively more of the metallic fraction has made it possible to obtain samples pure enough and large enough to examine in the mass spectrometer. It appears that two compounds may be present, a hydrocarbon $C_{10}H_{16}$, mol.wt. 136, and a compound with the empirical formula $C_8H_{14}O_2$, mol.wt. 142 with a CH_3CO fragment. It was suggested in an earlier paper that the metallic flavoured compound might be a C_8 α-dicarbonyl and such a compound as the 2,4-dinitrophenylhydrazone has been isolated from the metallic flavour fraction from washed cream. However, flavour tests with synthetic C_8 dicarbonyl compounds have not revealed any with a metallic flavour.

The chromatograms from a number of butters and butterfats are depicted diagrammatically for purposes of comparison in Fig. 1. It will be seen that the chromatogram for fish-oil flavoured butter (*c*, *d*) from washed cream differed only slightly from that for the fish-oil flavoured butterfat previously studied (*e*), the main difference being the lower contents of hex-2-enal, hepta-2,4-dienal, and non-2-enal, and the higher contents of hept-2-enal, and n-octanal.

Comparison of chromatograms for butters from fish-oil flavoured washed cream immediately after churning (Fig. 1, *c*) and after 9 days (*d*), revealed an increase in the n-octanal, oct-2-enal, n-nonanal and non-2-enal peaks, which corresponds well

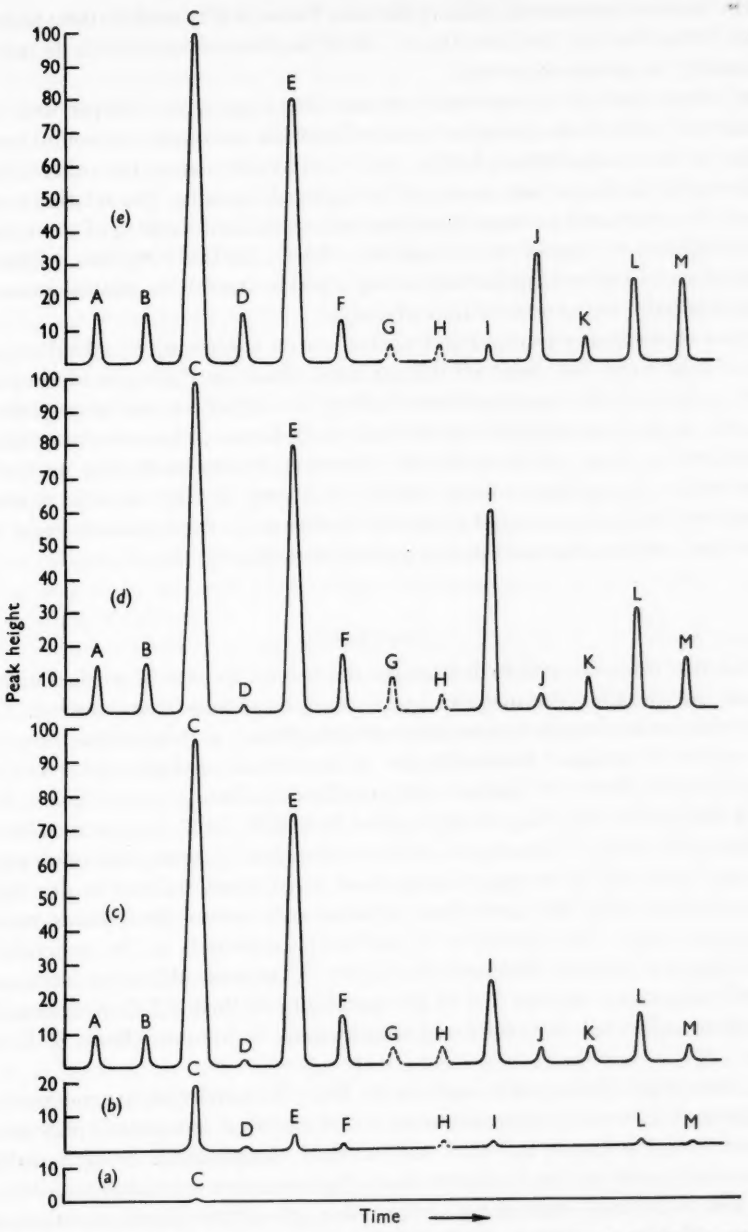

Fig. 1. Diagrammatic gas chromatograms of volatile carbonyl compounds in 1 kg quantities of: (*a*) fresh butters made from unwashed and washed cream containing no added copper or ascorbic acid; (*b*) fresh butter made from unwashed cream containing added copper and ascorbic acid; (*c*) as for (*b*) but made from washed cream; (*d*) as for (*c*) but 9 days old; (*e*) butterfat treated with copper, NDGA, and citric acid, 3 months old (Forss *et al.* 1960*a*). A, n-pentanal; B, pent-2-enal; C, n-hexanal; D, hex-2-enal; E, n-heptanal; F, hept-2-enal; G, 'mushroom' compound; H, 'metallic' compound; I, n-octanal; J, hepta-2,4-dienal; K, oct-2-enal; L, n-nonanal; M, non-2-enal.

with the observed increase in tallowy flavour. Tests on fifty such butters have shown that on further holding for 3 months at −15 °C the flavour compounds do not change significantly in nature or amount.

The butter made from unwashed cream containing added copper and ascorbic acid showed, when fresh, a concentration of metallic flavoured compound approaching that of the washed cream butter, but considerably less of the other aldehydes. The flavour of the butter was, as would be expected, metallic. The relative amount of the metallic compound increased to an amount equivalent to 90 % of the n-heptanal peak on holding the butter for 5 weeks at −15 °C. At this stage the heights of the n-hexanal and n-heptanal peaks were about equal to those from washed cream butter treated similarly and examined immediately.

Butters made from unwashed and washed cream containing no added copper and ascorbic acid were free from off-flavour when fresh and the gas chromatograms (Fig. 1, *a*) showed only trace amounts of aldehydes. After 6 weeks' storage at −15 °C the butter made from unwashed cream had no off-flavour. The amounts of aldehydes had doubled but were still insignificant. However, butter made from washed cream after 6 weeks' storage had a stale, tallowy and very slightly metallic flavour, and the carbonyl content was equal to that of butter made from washed cream treated with copper and ascorbic acid and examined immediately (Fig. 1, *c*).

DISCUSSION

Of the two elements which distinguish the fish-oil flavour in washed cream, the oily and the metallic, the metallic has not yet been fully characterized. The oily component was due largely to n-hexanal and n-heptanal. n-Hexanal has been reported in a number of oxidized foodstuffs (for example heat-oxidized cottonseed oil, by Swift, O'Connor, Brown & Dollear, 1949; oxidized chicken flesh by Pippen, Nonaka, Jones & Stitt, 1958; rancid pork fat by Gaddis & Ellis, 1957; and gamma-irradiated meat fat by Witting & Schweigert, 1958). n-Heptanal however, although stated by Tollenaar (1949) to be a major component in oxidized flavour in dry butterfat and dried whole milk, has rarely been reported and was not for instance mentioned in a recent review 'The formation of carbonyl compounds in the autoxidation of olefinic fats' by Täufel & Zimmermann (1959). That these aldehydes have not been reported more often may be due to the instability of their 2,4-dinitrophenylhydrazones when subjected to certain chromatographic techniques (Forss & Dunstone, 1957).

Van Duin of the Netherlands Institute for Dairy Research (private communication) has suggested that non-conjugated unsaturated carbonyl compounds play an important part in trainy flavour in butter and oxidized phospholipid. Some, possibly two, of these compounds as the 2,4-dinitrophenylhydrazones were eluted with the n-pentanal and n-hexanal derivatives from silica gel-nitromethane chromatographic columns. Hoffmann (1960) of the Unilever Research Laboratory, Vlaardingen has isolated small amounts of hex-*cis*-3-enal from reverted soybean oil where it makes an important contribution to the flavour.

The fact that the flavours produced in butterfat by the addition of pentanal, hexanal and heptanal derived from oxidized fat sometimes differed from the flavours

produced by the synthetic compound may have been due to the presence of trace amounts of such compounds as the 3-enals.

Fish-oil flavour whether induced in butterfat by NDGA and citric acid or in washed cream by the addition of copper and ascorbic acid arises from the same carbonyl compounds. In the butterfat, a single-phase system, there is an antioxidant, an acid, and a minimum content of phospholipid (0·025 %). In the washed cream there is a separate water phase, a phospholipid-protein film at the fat-water interface, an oxidant, probably concentrated in the film, and again acid conditions.

The recent work in the Netherlands indicates that the presence of fat globule membrane material, in which phospholipid is probably the key constituent, is essential for the development of fishy flavour in washed cream, and our own observations confirm this. With normal cream, however, where the concentration of phospholipid is at least as high, oxidation by the same agencies produces less of the oily-flavoured components so that the flavour is metallic. In skim-milk (Forss, Pont & Stark, 1955), which contains small amounts of phospholipid as well as fat, the addition of copper and either ascorbic acid or hydrogen peroxide results in the development of a cardboard flavour, due mainly to the 2-enals and 2,4-dienals. If, further, we may take the fish-oil flavour in cold-stored unsalted butter as probably due to the same flavouring compounds as the defect in washed cream, we have similar compounds arising from acid and pro-oxidant conditions again in a two-phase system with phospholipid present.

No clear picture of the compounds which provide material for the development of fish-oil flavour, or of the range and the limits of the conditions under which it may develop, appears from these observations. The parent material is probably the more highly unsaturated fatty acids, but whether these may act as sources in both triglyceride or phospholipid is not clear. The variation in the relative amounts of the flavouring compounds according to the presence or absence of such substances as copper, NDGA, ascorbic acid, citric acid, a water phase and the constituents which go with it in skim-milk, strongly suggests that the atomic environment of the oxidizing double-bond, or perhaps more important, the decomposing fatty acid peroxide, influences the relative amount of the various compounds which are formed.

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The volatile compounds associated with tallowy and painty flavours in butterfat

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Summary. From butterfat with tallowy and painty flavours, the same range of carbonyl compounds was obtained as from fishy flavoured butterfat or washed cream. They consisted of the C_{5-10} n-alkanals, the C_{5-10} alk-2-enals, hepta-2,4-dienal and a compound with a metallic flavour. Heptan-2-one, present only in small amounts in washed cream, was also isolated. Three factors distinguished the tallowy and painty from the fish-oil flavoured butterfat. There was a relative increase in n-heptanal, n-octanal, n-nonanal, heptan-2-one, hept-2-enal and non-2-enal in the tallowy butterfat and a relative increase in n-pentanal and the C_{5-10} alk-2-enals in the painty butterfat. Secondly, the 'metallic' compound was present in such small relative amounts in both the tallowy and painty flavoured butterfats as to have no effect on the flavour. Thirdly, the total weight of volatile carbonyl compounds was about 10 times greater in the tallowy and 100 times greater in the painty butterfat than in the fish-oil flavoured butterfat.

The many investigations that have been made into the volatile compounds associated with oxidized flavours in foods have led to general agreement that all the important ones are carbonyl in nature (see, for instance, the recent review of Täufel & Zimmermann, 1959). It is important not only to identify carbonyl compounds occurring in an oxidized foodstuff, but to know the amount of each compound and its contribution to the over-all flavour. These have been the aims of investigations of oxidized dairy products in this laboratory. Two earlier studies dealt with fish-oil flavour in butterfat (Forss, Dunstone & Stark, 1960*a*) and fish-oil flavour in washed cream (Forss, Dunstone & Stark, 1960*b*). This paper describes the characterization of the compounds associated with tallowy and painty flavours in butterfat, and the determination of the contribution of these compounds to the flavours.

Shipstead & Tarassuk (1953) had suggested that n-heptanal, n-octanal and n-nonanal might be responsible for tallowy flavour in whole milk powder, while Day & Lillard (1960) isolated the C_{1-10} n-alkanals and the C_{4-11} alk-2-enals from phospholipid-free butterfat exposed to diffuse light at room temperature. Kawahara, Dutton & Cowan (1952) isolated pent-2-enal from a 'painty fraction' obtained from autoxidized methyl linolenate.

EXPERIMENTAL

(1) *Sources of tallowy and painty butterfat*

Commercial butterfat (Coombs, 1956) was used as raw material. Cupric acetate dissolved in propylene glycol was added to butterfat to give a concentration of 0·5 ppm. copper. Tallowy flavours were induced by storing this butterfat in open containers in an oven at 45 °C for periods of 2–10 weeks. On prolonged holding under these conditions the flavour changed to painty or drying oil. Such flavours also developed when butterfat was placed in open glass flasks in the laboratory, more rapidly when copper had been added. The samples of painty flavoured butterfat examined were 1–2 years old, were bleached and smelt like oxidized linseed oil.

(2) *Distillation from butterfat*

The method for distilling the flavouring compounds from the butterfat has been described previously (Forss *et al.* 1960*a*).

(3) *Characterization of flavouring compounds*

The methods for the isolation and identification of the volatile compounds, using chiefly gas chromatography, absorption spectrophotometry and study of the 2,4-dinitrophenylhydrazones, were similar to those used in the study of fish-oil flavour (Forss *et al.* 1960*a*, *b*).

Measurements of the quantity of each compound were based on the areas of peaks recorded in their separation by gas chromatography; the percentage of carbonyl represented by each peak was determined by preparation of the 2,4-dinitrophenylhydrazone. Butterfats containing single aldehydes or mixtures were prepared for flavour assessment from aldehydes isolated from tallowy and painty flavoured butterfats, or from synthetic compounds purified by gas chromatography.

RESULTS

(1) *Preliminary observations*

The characteristic odours of the steam distillates from tallowy and painty flavoured butterfats were unchanged when treated with N-H_2SO_4, but were destroyed by N-H_2SO_4 containing 2,4-dinitrophenylhydrazine, indicating that the odours were due to carbonyl compounds. This was further confirmed when the light petroleum extract of the tallowy distillate was shaken with a solution of 2,4-dinitrophenylhydrazine in N-H_2SO_4. Examination of the concentrated light petroleum extract on a silicone oil gas chromatographic column showed no peaks, indicating that not only was the odour due to carbonyl compounds but that the volatile compounds were entirely carbonyl.

Butterfats with tallowy and painty flavours were distilled, following the quantitative method of Machulis (1957). The distillates were examined in a spectrophotometer over the range of 210–300 mμ. The optical densities at the light absorption maxima (λ_{max}) are listed in Table 1. Two wavelength ranges were chosen—that at 215–223 mμ which represents mainly the alk-2-enals, and that at 267–281mμ representing a

combined effect of n-alkanals and the alka-2,4-dienals. (Measurement in these ranges could provide a picture of oxidation in fats, perhaps as useful and simpler than that suggested by Henick, Benca & Mitchell (1954) in which the absorption of alkaline solutions of 2,4-dinitrophenylhydrazones is measured at 430 and 460 mμ.) For comparison, corresponding data are given for an untreated butterfat stored in a sealed can at the same temperature and for the same time as the tallowy butterfat, for butterfat treated with copper, NDGA and citric acid (Forss *et al.* 1960*a*) and stored under the same conditions as the untreated butterfat, and for butter obtained from washed cream treated with copper and ascorbic acid (Forss *et al.* 1960*b*). It will be seen that absorption in the range characteristic of the alk-2-enals, and n-alkanals and alka-2,4-dienals is much greater in the tallowy than in the fish-oil flavoured butterfats. Further, the absorption due to these aldehydes is much greater in the painty than in the tallowy butterfat. This corresponds with the amounts of total volatile carbonyl compounds isolated (Table 1).

Table 1. *Spectrophotometric measurements on distillates from oxidized butterfats*

Treatment of butterfat	Flavour	Total volatile carbonyls, mg/kg butterfat	Optical density in a 1 cm cell at λ_{max} (wavelength in mμ)
Untreated, stored at 45 °C in sealed opaque container for 4 weeks	Very slightly oily and tallowy	—	0·062 (279)*
Obtained from washed cream treated with copper and ascorbic acid, examined after churning	Strong fish-oil	1·5	0·074 (281), 0·091 (215)*
Treated with copper, NDGA and citric acid, stored at 45 °C in sealed opaque container for 4 weeks	Strong fish-oil	1·5	0·160 (270), 0·131 (222)*
Treated with copper, stored at 45 °C in an open opaque container for 4 weeks	Strong tallowy	15	0·805 (267), 1·120 (225)
Treated with copper and stored at room temperature in the light for 2 years	Strong painty	125	3·52 (275), 7·36 (223)†

* Measured in a 4 cm cell. † Diluted 1:20 for measurement.

(2) *Isolation and identification of compounds*

Light petroleum extracts of the steam distillates from butterfats with tallowy and painty flavours were fractionated on temperature-programmed gas chromatographic columns with silicone oil and 'Carbowax 400' as stationary phases, compounds being collected for odour and flavour appraisal and for identification through their 2,4-dinitrophenylhydrazones.

As in the study of fish-oil flavour in butterfat the C_{5-9} n-alkanals and alk-2-enals, heptan-2-one and hepta-2,4-dienal represented 90% of the volatile carbonyl compounds. Small amounts were also isolated of n-decanal and dec-2-enal, together

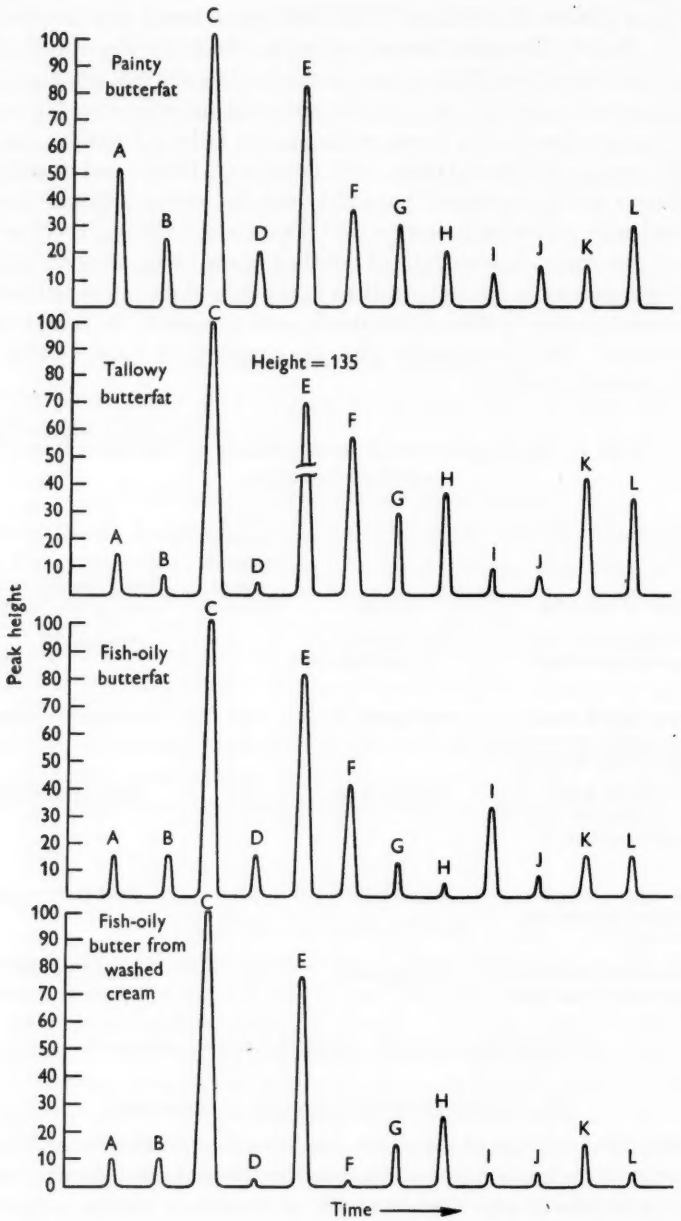

Fig. 1. Diagrammatic gas chromatograms of volatile carbonyl compounds in fish-oil flavoured butter made from washed cream, and in butterfats with fish-oil, tallowy and painty flavours. Peak heights of each chromatogram have been adjusted to n-hexanal = 100, to show relative amounts of various carbonyl compounds associated with the different flavours. A, n-pentanal; B, pent-2-enal; C, n-hexanal; D, hex-2-enal; E, n-heptanal; F, heptan-2-one; G, hept-2-enal; H, n-octanal; I, hepta-2,4-dienal; J, oct-2-enal; K, n-nonanal; L, non-2-enal.

with a compound corresponding in all its characteristics except melting-point to octa-2,4-dienal (Tables 2 and 3; Forss *et al.* 1960*a*).

The identity of heptan-2-one was confirmed by measuring the infra-red spectra of 2,4-dinitrophenylhydrazones as KBr disks and comparing them with the spectra of the heptan-2-one and heptan-4-one derivatives (see Ross, 1953).

Fig. 1 shows gas chromatograms obtained from flavour extracts of oxidized lipids with painty, tallowy and fish-oil flavours. It indicates the relative amounts of the various carbonyl compounds within each of the flavour extracts. Peak heights of each chromatogram have been adjusted to n-hexanal at 100 on the vertical scale.

While peaks for the 'metallic' compound have not been shown in Fig. 1 there

Table 2. *Experiments to reproduce tallowy flavour by adding gas chromatographic fractions to butterfat*

Expt. no.	Compounds in fraction	Comments on flavour
1	C_{5-10} n-alkanals, C_{5-10} alk-2-enals, heptan-2-one, hepta-2,4-dienal and 'octa-2,4-dienal'	Tallowy
2	n-Pentanal and pent-2-enal	Slightly painty
3	n-Hexanal, n-heptanal, heptan-2-one, hex-2-enal, hept-2-enal and 'metallic' compound	Oily, like crushed ants, tallowy
4	n-Octanal, n-nonanal, oct-2-enal, non-2-enal and 'octa-2,4-dienal'	Cucumber and tallowy
5	n-Decanal and dec-2-enal	Slightly cucumber and tallowy
6	Non-2-enal	Cucumber
7	n-Octanal, n-nonanal, oct-2-enal and 'octa-2,4-dienal'	Tallowy
8	As for no. 1 but without n-heptanal and heptan-2-one	Tallowy
9	n-Heptanal and heptan-2-one	Oily, like crushed ants

Table 3. *Experiments to reproduce painty flavour by adding gas chromatographic fractions to butterfat*

Expt. no.	Compounds in fraction	Comments on flavour
1	C_{5-10} n-alkanals, C_{5-10} alk-2-enals, heptan-2-one, hepta-2,4-dienal and 'octa-2,4-dienal'	Painty
2	n-Pentanal and pent-2-enal	Painty
3	C_{6-9} n-alkanals, C_{6-9} alk-2-enals, heptan-2-one, hepta-2,4-dienal and 'octa-2,4-dienal'	Like green leaves, tallowy, painty, alk-2-enals
4	n-Hexanal, n-heptanal, heptan-2-one, hex-2-enal and hept-2-enal	Tallowy, painty
5	n-Octanal, n-nonanal, oct-2-enal, hepta-2,4-dienal and 'octa-2,4-dienal'	Tallowy
6	Non-2-enal	Cucumber
7	As for no. 5 plus non-2-enal	Tallowy, cucumber
8	As for no. 3 plus n-decanal and dec-2-enal	Tallowy, slightly painty

was relatively little of this flavour in tallowy and painty flavoured butterfats. The weights of volatile carbonyl compounds in the three types of oxidized fats are listed in Table 1.

These carbonyl compounds collected from gas chromatographic fractionations of extracts from tallowy and painty flavoured butterfats were added singly and in groups to bland butterfat. The flavours were observed and compared with those given by the same compounds derived from other sources (Tables 2 and 3).

DISCUSSION

The purpose of this investigation was to give a quantitative picture of the carbonyl compounds isolated and so make it possible to determine the importance of each compound in the particular flavour. The results show that it is the relative and total amounts of the carbonyl compounds that determine whether the flavour of a butterfat is tallowy or painty.

Three factors distinguish a butterfat with tallowy flavour from the butterfat with fish-oil flavour previously studied. First, as can be seen in Fig. 1, there is relatively a higher content of n-heptanal, n-octanal, n-nonanal, heptan-2-one, hept-2-enal and non-2-enal in the tallowy butterfat. Secondly, the 'metallic' compound was present in relatively much smaller amount, as shown by comparative flavour tests, and thirdly the total amounts of volatile carbonyl compounds in tallowy butterfat was about 10 times greater.

The same three factors distinguish butterfat with a painty flavour from butterfat with a fish-oil flavour. Fig. 1 shows that n-pentanal and the C_{5-9} alk-2-enals are in higher concentration in painty flavoured butterfat. The 'metallic' compound was in even smaller relative amount than in the tallowy butterfat and its influence negligible. The total amount of volatile carbonyl compounds was also much greater, about 100 times that in butterfat with fish-oil flavour.

Flavour tests (Tables 2 and 3) confirmed the hypothesis advanced previously (Forss *et al.* 1960*b*) that n-octanal and n-nonanal provided elements in the tallowy flavour while n-pentanal and pent-2-enal were important in the painty flavour. This was shown most effectively by observing the change in flavour on removing these compounds from the flavour extract. Close reproduction of the tallowy and painty flavours was obtained only when all of the compounds already mentioned were added to bland butterfat.

Day & Lillard (1960), working with 2,4-dinitrophenylhydrazones, have reported that n-pentanal, n-hexanal, n-heptanal and non-2-enal occurred each in about equal amounts in butterfat exposed to diffuse light. These four aldehydes represented 70% of the monocarbonyl compounds, which is in line with our findings with painty butterfat. However, they reported only traces of alkan-2-ones, whereas we isolated appreciable amounts of heptan-2-one. That this ketone was not derived from heating of the butterfat during manufacture was shown by its almost complete absence in the unoxidized butterfat. It is interesting to note that Schepartz & Daubert (1950) isolated its isomer, heptan-4-one from the reversion compounds in hydrogenated soybean oil, while Keeney & Doan (1951) described the 2,4-dinitrophenylhydrazone of a C_7 alkanone (possibly heptan-2-one) isolated from oxidized milk fat.

Day & Lillard did not isolate any alka-2,4-dienals, and have concluded that these compounds were derived from phospholipid. We isolated two alka-2,4-dienals but they may have been derived from the traces of phospholipid (0·025 %) in our butterfat.

The authors express their thanks to Mr J. F. Horwood for infra-red determinations carried out, and Mr G. Loftus Hills of the Dairy Research Section, C.S.I.R.O., for helpful advice and discussion.

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The effect of various sampling intervals on the estimation of lactation milk yield and composition

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SUMMARY. A study was made of the effect of various sampling intervals on the accuracy of estimating lactation milk yield, fat yield, solids-not-fat (S.N.F.) yield, fat percentage and S.N.F. percentage. The basic data consisted of daily milk, fat and S.N.F. yields for eighteen lactations made by twelve Dairy Shorthorn cows. Estimates of the lactation yields and quality were obtained using sampling intervals of 7, 14, 28, 42, 56 and 63 days, and were expressed as differences from the actual values. Only in the case of milk yield was there any effective bias in the estimates. This bias increased with increasing sampling interval.

The distributions of differences between estimated and actual values are presented. The error of estimation increased with increasing sampling interval. Keeping in mind the comparatively small-sized sample used in the investigation, the results are discussed in the light of the purposes for which records are kept.

With few exceptions the production of individual dairy cows is estimated from the results of a limited number of weighings and chemical analyses carried out at approximately equal intervals throughout lactation (Ashton, 1956). The yield and composition of milk fluctuates considerably from day to day, and the estimates of lactation production are therefore subject to error. Many studies of the magnitude of the error involved in estimating yield and fat content at various sampling intervals have been carried out and an early review by the International Institute of Agriculture (1935) has been brought up to date by Smith in an addendum to Ashton's (1956) work.

The computations relevant to such studies, although exceedingly simple in themselves, are very laborious and repetitive; thus electronic computers are particularly well suited for handling them and permit rather more detailed treatment than hitherto. An opportunity arose to analyse daily records for eighteen lactations using the Elliott N.R.D.C. 401 electronic computer at Rothamsted, and in this paper the effects of using various sampling intervals on the accuracy of estimating lactation milk yield, fat yield, S.N.F. yield, fat percentage and S.N.F. percentage are described. Despite the small number of lactations involved, the analysis would have been too time-consuming to be practical without the aid of electronic machinery.

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SOURCE OF DATA

The data used consisted of daily milk, fat and S.N.F. yields for each of eighteen lactations made by twelve Dairy Shorthorn cows in the herd of the Cumberland and Westmorland Counties Farm School. The eighteen lactations included five second, five third, three fourth, four fifth and one sixth lactation records. The few missing records were estimated by averaging the two previous and two subsequent days' figures. To conform with standard recording practice in England and Wales, the first 4 days after calving were regarded as a suckling period and the relevant records discarded and the lactations were amputated at 305 days. The numerical information contained in the daily records was transferred to punched cards for processing by the electronic computer.

BASIC COMPUTATIONS

For each lactation, the total yields of milk, fat and S.N.F. were obtained by summing the daily yields: the average fat and S.N.F. percentages were calculated from these totals. These measures of actual lactation production provided standards for judging the relative accuracy of the various estimates.

In each lactation samples were taken at 7-, 14-, 28-, 42-, 56- and 63-day intervals, and estimates of the lactation yields and average composition calculated. Every possible estimate was obtained at each sampling interval so that, for each lactation, there were seven estimates of each of the five measures of yield and quality at the 7-day sampling interval, fourteen at the 14-day interval and so on.

In estimating lactation milk yield, the yields on the first and last sample days were multiplied by $[d+\frac{1}{2}(I+1)]$, where d is the number of days between the end of the suckling period and the first sample day, or between the last sample day and the end of the 305-day lactation, and I is the sampling interval. For sampling intervals with an even number of days this multiplier was rounded up to a whole number which resulted in a slight, but in practice unimportant, tendency to overestimate the lactation yield in these cases. The yields on the remaining sample days were multiplied by the sampling interval and the whole summed to give the lactation estimate. The estimates of lactation fat percentage and S.N.F. percentage were calculated from the sums of the yields of fat or S.N.F. and the sum of the milk yields on each sample day, and the lactation fat and S.N.F. yields were estimated from these percentages and the actual lactation milk yield.

All analyses were carried out on the differences between the estimated values and the appropriate actual lactation figures.

BIAS IN ESTIMATION

The mean differences between estimated and actual lactation values for each sampling interval, correct to two decimal places, are shown in Table 1.

For fat and S.N.F. yields and percentages the biases are negligible, the differences between the estimated and actual lactation values at each sampling interval being effectively distributed about zero. In the case of milk yield, however, the mean difference increases with increasing sampling interval from +2 gal for the 7-day interval to +41 gal for the 63-day interval.

For a given sampling interval, the variances within lactations were not homogeneous and therefore it was not strictly possible to use the variance ratio to test the significance of variations in the mean difference between lactations. However, when the smallest variance within individual lactations for each sampling interval

Table 1. *The mean differences between estimated and actual lactation values (estimated minus actual) correct to 2 decimal places*

Sampling interval, days	Milk yield, gal	Fat yield, lb	S.N.F. yield, lb	Fat, %	S.N.F., %
7	+ 2·31	0·00	0·00	0·00	0·00
14	+ 5·36	+0·02	+0·01	0·00	0·00
28	+14·42	+0·05	+0·09	0·00	0·00
42	+24·75	−0·06	+0·21	0·00	0·00
56	+34·13	+0·12	+0·39	0·00	0·00
63	+40·57	+0·21	+0·12	0·00	0·00
Mean actual lactation production	883	328	787	3·75	8·92

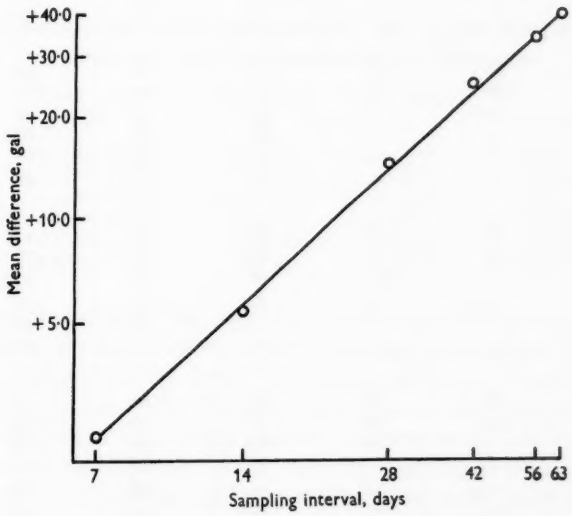

Fig. 1. The relationship between the bias in estimating lactation milk yield and the sampling interval. Log scales.

was used as an estimate of error, none of the between lactations mean squares was found to be significant; hence the positive bias in the estimation of milk yield and the lack of bias in the estimation of fat and S.N.F. yields and percentages may be said to be consistent for each lactation. The relationship between log mean difference for milk yield and log sampling interval was linear over the range of sampling interval studied (Fig. 1).

There was no indication that the interval between the end of the suckling period and the first sample day affected the bias in estimation.

DISTRIBUTION OF DIFFERENCES

The percentages of estimates falling within various ranges of the true lactation values are shown in Table 2 for fat and S.N.F. where the differences are effectively distributed about zero and in Table 3 for milk yield where the positive bias has been taken into account. As would be expected, the accuracy of estimation decreases with increasing sampling interval.

Table 2. *The percentages of estimates falling within various ranges of the true lactation values (fat and S.N.F. yields and percentages)*

	Sampling interval, days					
Range	7	14	28	42	56	63
Fat yield, lb						
±10	94	82	67	56	51	48
±20	100	98	92	82	80	73
±30	—	100	98	94	91	87
±40	—	—	100	97	96	94
±50	—	—	—	99	98	98
±60	—	—	—	—	99	99
S.N.F. yield, lb						
±10	95	90	76	70	62	63
±20	100	99	94	90	87	88
±30	—	—	98	96	95	94
±40	—	—	99	98	97	97
±50	—	—	—	99	98	98
±60	—	—	—	—	99	99
Fat, %						
±0·1	93	73	61	50	45	41
±0·2	100	97	88	78	74	67
±0·3	—	100	97	91	87	84
±0·4	—	—	99	97	94	92
±0·5	—	—	—	99	98	97
±0·6	—	—	—	—	99	99
S.N.F., %						
±0·1	94	86	70	65	54	57
±0·2	100	98	94	89	85	84
±0·3	—	99	98	96	94	93
±0·4	—	—	99	98	97	96
±0·5	—	—	—	99	99	98
±0·6	—	—	—	—	—	99

Table 3. *The percentages of estimates of milk yield falling within various ranges of the true lactation values for different sampling intervals*

7-day		14-day		28-day		42-day		56-day		63-day	
Range, gal	%	Range, gal	%	Range, gal	%	Range, gal	%	Range, gal	%	Range, gal	%
−25, +25	93	−20, +30	76	−10, + 40	55	− 0, + 50	50	+ 10, + 60	36	+ 15, + 65	26
−50, +50	100	−45, +55	96	−35, + 65	84	− 25, + 75	81	→ 15, + 85	67	→ 10, + 90	54
		−70, +80	100	−60, + 90	96	− 50, +100	94	→ 40, +110	85	→ 35, +115	76
				−85, +115	99	− 75, +125	98	→ 65, +135	95	→ 60, +140	87
						−100, +105	99	→ 90, +160	97	→ 85, +165	94
								→115, +185	98	→110, +190	97
								→140, +210	99	→135, +215	99

The standard deviations of the differences between the estimated and actual lactation values for each sampling interval and the limits within which approximately 95% of the estimates should fall, assuming a normal distribution of the differences, are given in Tables 4 and 5. The latter figures are in good agreement with the actual distributions of the differences given in Tables 2 and 3, and the standard deviation can therefore be used as a general measure of the accuracy of estimation at a given sampling interval.

Table 4. *Standard deviations of the differences between estimated and actual lactation production*

Sampling interval, days	Milk yield, gal	Fat yield, lb	S.N.F. yield, lb	Fat, %	S.N.F., %
7	13·0	4·48	4·74	0·057	0·055
14	22·8	7·84	6·83	0·090	0·079
28	38·0	11·50	10·48	0·133	0·121
42	40·9	15·52	13·62	0·180	0·160
56	54·8	18·02	15·82	0·208	0·181
63	68·1	20·12	16·87	0·232	0·194

Table 5. *Limits within which approximately* 95% *of the estimates should fall assuming a normal distribution of differences*

Sampling interval, days	Milk yield, gal	Fat yield, lb	S.N.F. yield, lb	Fat, %	S.N.F., %
7	+ 28·3, −23·7	± 8·96	± 9·48	±0·11	±0·11
14	+ 51·0, −40·2	±15·68	±13·66	±0·18	±0·16
28	+ 90·4, −61·6	±23·00	±20·96	±0·27	±0·24
42	+106·6, −57·0	±31·04	±27·24	±0·36	±0·32
56	+143·7, −75·5	±36·04	±31·64	±0·42	±0·36
63	+176·8, −95·6	±40·24	±33·74	±0·46	±0·39

The relationships between log standard deviation of difference and log sampling interval for each of the five measures of yield and quality were linear over the range of sampling interval studied (Figs. 2–4). With one exception—yield at the 7-day interval—the standard deviation of the difference for fat was greater than that for S.N.F. but this is to be expected in view of the smaller variation in the latter characteristic.

The lactation yields of fat and S.N.F. were also calculated from the various estimates of average composition, and the estimates of milk yield obtained at the 7-day sampling interval, with a consequent reduction in the accuracy of estimation. The standard deviations of the differences for each sampling interval are given in Table 6. The error in estimating the lactation fat percentage and S.N.F. percentage increases with increasing sampling interval and the constant error introduced by basing the estimates of lactation fat and S.N.F. yields on the estimated instead of on the actual milk yield becomes relatively less important. Consequently, the differences between the standard deviations in Tables 4 and 6 become less as the sampling interval increases. At the shorter sampling intervals the error in estimating S.N.F. yield on this basis is considerably greater than the error in estimating fat yield because of the greater S.N.F. percentage (an average of 8·92% S.N.F. compared with 3·75% fat) which increases the effect of the error in estimating milk yield.

In the past, results have commonly been expressed in terms of the percentage

difference between estimated and actual lactation production. In order to provide comparable figures the standard deviations of the percentage differences for milk yield, fat percentage and S.N.F. percentage are given in Table 7. These increase linearly with increasing sampling interval, a relationship previously noted by Dick (1950) and others.

The interval between the end of the suckling period and the first sample day did not apparently affect the accuracy of estimation.

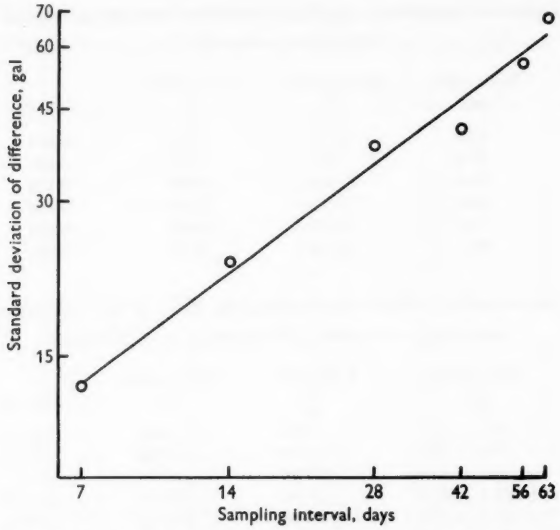

Fig. 2. The relationship between the standard deviation of the difference between estimated and actual lactation milk yield and the sampling interval. Log scales.

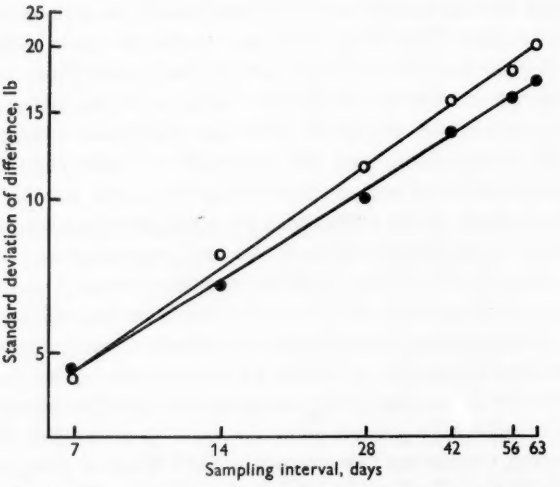

Fig. 3. The relationship between the standard deviation of the difference between estimated and actual fat yield, ○, and S.N.F. yield, ●, and the sampling interval. Log scales.

The lack of homogeneity of the mean squares within lactations for each sampling interval means that the effect of a given sampling interval on the accuracy of estimating lactation production is not necessarily the same for different cows or even different lactations of the same cow. This also results in a heterogeneity of the regressions of log standard deviation of difference on log sampling interval for each lactation showing that the change in accuracy with increasing sampling interval varies from cow to cow.

Table 6. *Standard deviations of the differences between estimated and actual lactation production for fat and* S.N.F. *yields using estimated milk yields based on the 7-day sampling interval*

Sampling interval, days	Fat yield, lb	S.N.F. yield, lb
7	7·90	12·46
14	9·99	13·39
28	13·05	15·56
42	16·69	17·81
56	19·02	19·17
63	21·01	19·95

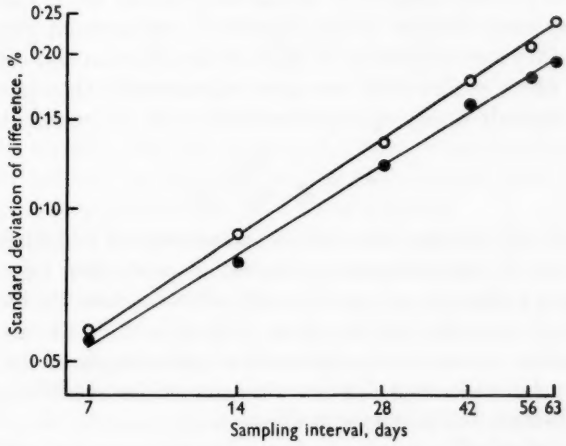

Fig. 4. The relationship between the standard deviation of the difference between estimated and actual fat percentage, ○, and S.N.F. percentage, ●, and the sampling interval. Log scales.

Table 7. *Standard deviation of the percentage differences between estimated and actual lactation milk yield, fat % and* S.N.F. %

Sampling interval, days	Milk yield	Fat %	S.N.F. %
7	1·46	1·52	0·61
14	2·50	2·40	0·88
28	3·94	3·55	1·35
42	4·48	4·76	1·78
56	5·96	5·44	2·02
63	7·68	6·07	2·17

DISCUSSION

The results of previous studies of the error in estimating lactation production from samples taken at various intervals are rather contradictory, but this is not surprising for two reasons. First, not all workers had available daily records and secondly, their results refer to different groups of cows, often kept under completely different conditions. It is clear that the size of day-to-day variations in the yield and quality of the milk of individual cows, which may be considerably affected by environment in the broadest sense, affects the error in estimation, and in the circumstances detailed comparisons with our own results would be of little value. It is sufficient that the standard deviations of the percentage differences for milk yield and fat percentage do not appear to be exceptional so that, despite the small number of lactations analysed, we may have some confidence in using our results to discuss the effect of various sampling intervals in relation to the uses of production records.

Milk records may be used as aids in: (1) day-to-day feeding and management; (2) the selection of male and female breeding stock: (3) the culling of females.

Frequent weighing of milk is an advantage in herd management both for rationing and the early detection of illness, but there is no need for such records to be part of an official scheme and their frequency and accuracy can be left to individual farmers whose needs may vary. The use of milk records for management purposes need not, therefore, enter into a consideration of their use for selection and culling.

The progress which can be made in a given characteristic through the selection of breeding stock depends largely upon its heritability (h^2) which may be written

$$h^2 = \frac{4\sigma_s^2}{\sigma_s^2 + (\sigma_w^2 + \sigma_e^2)},$$

where σ_s^2 and $(\sigma_w^2 + \sigma_e^2)$ are the observed variances between and within sire progeny groups. Any error in the estimation of lactation production (σ_e^2) will inflate the observed variance within sire groups $(\sigma_w^2 + \sigma_e^2)$ and will reduce the heritability by an amount dependent upon the relative values of σ_s^2, σ_w^2, and σ_e^2. In this context, therefore, the expression of errors of estimation as percentages of the true lactation production may be misleading. Rather they should be related to the variation between individuals in the character in question.

The accuracy of a bull's progeny test, considered as the probable superiority of test daughters which will be repeated in future daughters, may be expressed in terms of the number of test daughters, n, and the heritability as $n/\{n + (4 - h^2)/h^2\}$ or alternatively in terms of n and the ratio $(\sigma_w^2 + \sigma_e^2)/\sigma_s^2$ as $n/\{n + (\sigma_w^2 + \sigma_e^2)/\sigma_s^2\}$ (Robertson, 1955). This expression approaches a value of 1 as the information about the bull's breeding worth becomes more complete. The ratio of within and between sire variances is, in fact, the number of daughters required for a test with an accuracy of 0·5 and is a more meaningful measure of the relative ease of evaluating a bull than the heritability.

Taking the variance between lactations in our data as an estimate of σ_w^2 and assuming heritabilities of 0·3 for milk yield weighing at 7-day intervals and of 0·5 for fat and S.N.F. percentages testing at 42-day intervals, estimates of σ_s^2 and of σ_w^2 for daily weighing and testing were calculated (Table 8) and the values of h^2 and of $(\sigma_w^2 + \sigma_e^2)/\sigma_s^2$ for each sampling interval derived (Table 9).

In the case of milk yield, the heritability and the number of daughters required for a progeny test with an accuracy of 0·5 change little with increasing sampling interval. The decrease in accuracy of estimation is of greater consequence, however, for fat and S.N.F. percentages.

Table 8. *Estimated variances between sire progeny groups* (σ_s^2) *and within sire progeny groups for daily weighing and testing* (σ_w^2)

	σ_s^2	σ_w^2
Milk yield, gal	1874	22946
Fat, %	0·0104	0·0403
S.N.F., %	0·0071	0·0245

Table 9. *Estimates of heritability and of the ratio of within and between sire variances* $\{(\sigma_w^2+\sigma_e^2)/\sigma_s^2\}$ *for each sampling interval*

Sampling interval, days	Heritability			$(\sigma_w^2+\sigma_e^2)/\sigma_s^2$		
	Milk yield	Fat %	S.N.F. %	Milk yield	Fat %	S.N.F. %
1	0·30	0·82	0·90	12·2	3·9	3·5
7	0·30	0·77	0·82	12·3	4·2	3·9
14	0·30	0·71	0·75	12·5	4·6	4·3
28	0·29	0·61	0·61	13·0	5·6	5·5
42	0·28	0·50	0·50	13·1	7·0	7·0
56	0·27	0·44	0·44	13·8	8·0	8·1
63	0·25	0·40	0·41	14·7	9·0	8·7

In practice the sampling interval chosen must be a compromise between accuracy on the one hand and cost on the other. Although our results must be treated with reserve, they give some guide on the question of accuracy.

It is clear from Tables 2 and 3 that mistakes in culling will be made when lactation estimates are based on the longer sampling intervals. These will be reflected in a lower repeatability of the characteristics and the reduction will be greater for fat and S.N.F. percentages than for milk yield. As far as the selection of breeding stock is concerned our results suggest that a longer sampling interval will have little effect in the case of milk yield but will be more serious in the case of composition.

Under the official recording scheme in England and Wales milk is weighed either daily or weekly by the herd owner and samples for fat analysis are taken by a recorder six times a year, or more frequently if the farmer requests and pays for the additional tests. Though this frequency of weighing is clearly unnecessary for the estimation of lactation production it is probably justified as a management aid. The cost of testing for fat percentage at less than monthly intervals is generally prohibitive and the decline in heritability from the 28- to the 63-day interval, if it is a true reflexion of the facts, will lead to a slight but material reduction in the genetic selection differential of females selected for breeding, but the change in the number of daughters required for a progeny test is of little practical importance where all milk-recorded animals are also fat tested.

It appears then that the accuracy of the official recording scheme in England and Wales is reasonably satisfactory but that the frequency of weighing could, if necessary, be reduced from weekly to monthly intervals without seriously affecting the estimates

of lactation milk yield. Where the records are to be used primarily for progeny testing A.I. bulls we find, in common with Carré, Poly & Vissac (1959), that two-monthly sampling intervals for both yield and quality could be sufficient since the accuracy of the progeny test can be maintained by increasing the number of recorded daughters per bull by 20–25 %.

We are indebted to Dr A. L. Provan and his staff in the Central Laboratory of the M.M.B. for providing the data on which this study was based, and to the Cumberland and Westmorland Counties Farm School for providing the daily milk weights and samples.

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Measurements of non-ionic iodine in the milk of dairy cows following oral administration of labelled sodium iodide

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Summary. It has been shown that iodine present as iodide in milk can be quantitatively removed by passing the milk through a column of an anion-exchange resin in the chloride form. Any residual iodine is present as non-ionic species which are not removed by the resin.

This technique was used to determine the non-ionic iodine in milk at various times after the oral administration of a single dose of ^{131}I to dairy cows and also during a multiple-dose experiment. Non-ionic ^{131}I was present in milk 6 h after its administration and the fraction in this form varied considerably in milk from different cows, ranging from 1·2 to 14·5 % of the total ^{131}I content. The chemical form of the non-ionic iodine was not investigated.

In recent years, the availability of radioactive isotopes of iodine has facilitated studies of iodine metabolism in animals, particularly of thyroid function. Tracer techniques have also been utilized to study the secretion of orally administered iodine into the milk of lactating cows. Measurements of this type are of practical importance as ^{131}I is a fission product and has been detected in both human and animal thyroids after nuclear weapon tests (Robertson & Falconer, 1959). It may also be spread after a reactor accident such as occurred at Windscale in 1957 and contaminate milk supplies so that they may be temporarily unsafe for human consumption.

After the Windscale accident, Cosslett & Watts (1959) studied the removal of radioactive iodine from milk by passing it through an anion-exchange resin in the chloride form. They found that from 96 to 98 % of the iodine could be removed by this means and as no improvement was effected by increasing the residence time of milk in the resin column, they suggested that the residual iodine was present in a non-ionic form. Previous work by Glascock (1954) indicated that after administration of ^{131}I to a cow all the activity in the milk was present as iodide, and similar results were obtained with goats by Wright, Christian & Andrews (1955). On the other hand, however, Lengemann & Swanson (1957) and Garner (1958) detected varying amounts of protein-bound ^{131}I in the milk of cows, after oral administration.

The present work was undertaken to investigate the amount of non-ionic iodine in the milk of dairy cows at various times after oral administration of a single dose of

^{131}I as iodide. Milk samples were passed through columns of anion-exchange resin to remove the ionic (iodide) iodine and the activity of the effluent was compared with that of the untreated milk.

EXPERIMENTAL

Animals

The four cows used in the experiments were all Red Poll × Ayrshire crossbreds. Cows W 226 and U 116 were used in the first and second single-dose experiments respectively, and cows W 188 and W 137 were used in the multiple-dose experiments. After administration of ^{131}I, they were kept indoors and fed on an unvarying diet of hay and commercial dairy nuts, with water *ad lib.* They were normally machine-milked twice daily at 06.00 and 16.00 h.

The ^{131}I was administered to the cows as NaI (nominally carrier-free) absorbed on anhydrous sodium phosphate in gelatine capsules. The stable iodine content of the doses was less than 1 μg, which is negligible compared with the normal daily intake of approximately 7 mg by these cows. In the single-dose experiment 1 mc was administered, and in the multiple-dose experiment 2 mc daily.

Preparation of anion-exchange column

The anion-exchange resin used in these experiments was the strongly basic Deacidite FF, sieved to obtain the 30–50-mesh material. The columns were made from glass tubing of 1·5 cm i.d., drawn out at one end and plugged with glass wool. About 12 ml of resin were used, giving a bed length of 7 cm and a total capacity of 12 m-equiv. Columns were pretreated with 50 ml M-NaCl solution and washed with distilled water until the effluent was chloride-free. The rate of flow was controlled by compressing, with a screw clip, a rubber tube attached to the end of the column.

Treatment of milk

No preservative was added to the milk samples as Cosslett & Watts (1959) reported that the use of hydrogen peroxide for this purpose changed the chemical form of iodine in the milk. If samples could not be treated soon after milking, they were stored in a refrigerator. Immediately before a run, KI carrier (4 mg) in a few ml of water was added to each litre of milk, which was shaken to ensure thorough mixing. Milk was then added to the drained column by means of a pipette, so as not to disturb the surface of the resin. The head of milk was increased to about 20 cm and maintained by the occasional addition of milk from a separating funnel in which the cream was retained. A forerun of at least 150 ml was discarded before collecting 50 ml fractions for analysis.

Measurement of milk activity

To measure the activity of untreated milk samples and successive fractions of milk from the resin column, 5 ml aliquots were pipetted into cylindrical polythene containers and counted in a well-type NaI crystal. The crystal was operated in conjunction with a photomultiplier and single-channel pulse-height analyser, which was set to count the gamma activity in the ^{131}I photopeak. The over-all counting efficiency was 14 %. With untreated milk samples the standard deviation of succes-

sive determinations about the mean was less than $\pm 2\%$. The activity of the effluent was very much lower, and although the counting errors were larger they were still less than $\pm 5\%$. Corrections were applied for counter paralysis and radioactive decay.

RESULTS AND DISCUSSION

Some initial experiments were carried out to study the performance of resin columns using a milk sample from a cow receiving daily doses of ^{131}I as iodide. The effect of varying the rate of flow of milk through the column on the removal of activity was studied, and it was found that it was incomplete at flow rates above 5 ml/min. In subsequent experiments, flow rates were restricted to 2 ml/min or less.

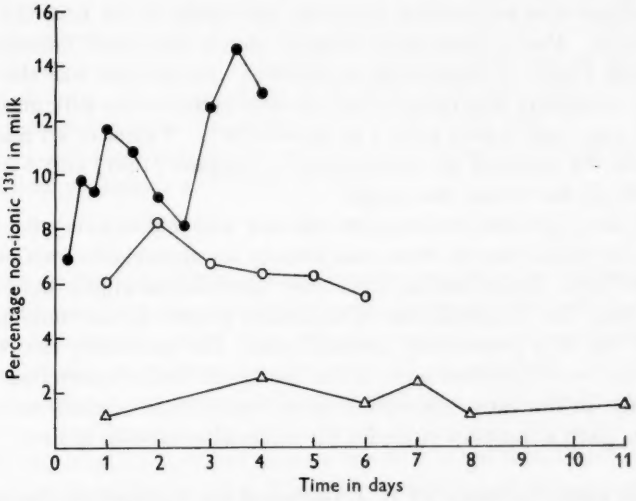

Fig. 1. Percentage of ^{131}I in milk in non-ionic form at various times after administration of tracer. ○—○, Single dose 3. xii. 59, cow W226; ●—●, single dose, 10. iii. 60, cow U116; △—△, multiple dose, 7. i. 60 *et seq.*, cows W188 and W137.

To check that the activity remaining in the effluent was due to non-ionic ^{131}I and not to incomplete removal of iodide, milk was passed through a column prepared as described above. As the initial effluent from the column was diluted with water, a forerun of 200 ml was discarded before collecting sixteen 50 ml fractions. These were analysed individually for ^{131}I and the activity was $4{\cdot}9 \pm 0{\cdot}1\%$ of that in the untreated milk. These fractions were combined and passed through a second column, identical with the first, and the activity of the effluent was the same as that from the first column, showing that little or none of the residual ^{131}I was removed. Two days later, some of the original milk which had been stored in a refrigerator was passed through a third column and the activity was $4{\cdot}8 \pm 0{\cdot}1\%$ of the untreated sample, showing that storage had not significantly affected the ionic/non-ionic ratio of the iodine in the milk. Identical results were obtained with and without the addition of KI carrier, but it was used in all subsequent experiments as an additional safeguard against adsorption of active material on surfaces.

As this method appeared to provide a sensitive measure of the relative amounts of ionic and non-ionic iodine in milk, it was used to study the distribution of ^{131}I between these forms, after its administration to lactating cows. The specific activity of milk samples in the single-dose experiments diminished rapidly after the first day with an approximate half life of 1·2 days, so that accurate measurements of treated milk were not possible after the 6th day. With the multiple-dose experiment, measurements were made over 2 weeks as the activity of the milk did not vary much.

The results of these various experiments are shown in Fig. 1. In the multiple-dose experiment, the milk analysed was a mixture from two cows dosed simultaneously and the non-ionic ^{131}I varied from 1·2 to 2·6 % of the total. In the single-dose experiments the proportion was higher, ranging from 5·5 to 14·6 % of the total. It is unlikely that this variation can be attributed to the different regimens, as the first day of a multiple-dose experiment is exactly equivalent to the first day of a single-dose experiment. Also in preliminary trials a value of 4·9 % was obtained in a single sample of milk from a multiple-dose experiment. As the diet was the same in all experiments, it appears that the level of non-ionic iodine in the milk must depend on the cow and may vary widely from 1 to at least 15 %. Values of 4·7 and 13·1 % for protein-bound ^{131}I reported by Lengemann & Swanson (1957) and 5 and 10 % by Garner (1958) all fall within this range.

In one of the single-dose experiments the cow was milked every 6 h for the first day and in the initial sample there was already an appreciable fraction of the ^{131}I in a non-ionic form. Apart from an initial rise, there did not appear to be any definite trend with time. The chemical form of the iodine present in non-ionic form was not investigated but it is presumably protein-bound. The possibility that the strongly basic resin may have liberated some of the less firmly bound iodine from the protein seems unlikely, as flow rates, corresponding to a wide range of residence times in the resin column, gave a constant value for the removal of labelled iodine.

The author wishes to thank Mr R. J. Garner of the Agricultural Research Council Radiobiological Laboratory, Compton, Berks, in whose department the cows were maintained and without whose co-operation this work would not have been possible.

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The colloidal phosphate of milk

III. Nature of its association with casein

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Summary. Removal of colloidal phosphate leads to changes in the properties of milk of which the increased viscosity and greatly increased sensitivity to calcium salts, and the apparently diminished degree of mutual integration of the various casein fractions are the most striking. Re-introduction of colloidal phosphate restores incompletely and only in certain instances the original properties of the milk.

These observations are interpreted as favouring the conception of chemical links between colloidal phosphate and casein in milk.

Recent work on the colloidal phosphate, or more properly the colloidal phosphate-citrate,* of milk has been concerned more with its composition (Pyne & McGann, 1960; Mulder & Schipper, 1959) than with the nature of its association with the casein, although the two matters are naturally not unconnected. In reviewing the subject some years ago ter Horst (1947) concluded that the evidence then available left a decision open between the alternatives of physical protection of colloidal phosphate by casein and a chemical union of the two. What little direct experimental evidence has since been forthcoming (Evenhuis & de Vries, 1956*a*) appears to favour the former alternative.

Colloidal phosphate, nevertheless, affects so many of the properties of casein in milk—for instance, its sensitivity to calcium and its coagulability when acted upon by rennet or by heat—that one hesitates to accept the very passive role usually ascribed to it. It constitutes, moreover, no negligible constituent of the casein micelles in milk, being present in all these micelles, irrespective of their dimensions, and providing some two-thirds of their calcium content. These considerations, and others to be discussed later, appeared to warrant the present reassessment of the role of colloidal phosphate in milk.

EXPERIMENTAL MATERIALS AND METHODS

Fresh mixed morning milk from the College Dairy Shorthorn herd was used throughout after removal of fat.

(*a*) *Colloidal phosphate-altered milks.* (1) Colloidal phosphate-reduced milks were prepared by controlled acidification of milk with concentrated HCl at 0 °C, followed

* For brevity the term colloidal phosphate will be used in this paper.

by prolonged dialysis against a considerable excess of the original milk (Pyne & McGann, 1960). (2) Colloidal phosphate-increased milks were prepared by appropriate alkalization of milk (by addition of up to 2 % of 2 N-NaOH at 0 °C with mechanical stirring), followed by dialysis for 96 h at 0–5 °C against 24 h changes of some twenty volumes of the original milk.

(*b*) *Soluble phosphate-low milks* were prepared by dialysis of milk for 24 h at 0 °C against forty volumes of a solution at pH 6·7–6·8 containing 4 m-mole/l. $CaCl_2$, 1 m-mole/l. $MgCl_2$, 75 m-mole/l. NaCl, 5 m-mole/l. sodium maleate and 5 % lactose. Milk thus treated still contained about 90 % of its original content of colloidal phosphate.

(*c*) '*Synthetic milk.*' This type of milk was prepared by simultaneously adding to colloidal phosphate-free milk at about 0 °C the amounts of calcium chloride, sodium phosphate, citrate and hydroxide required to re-form the original content of colloidal phosphate. In a typical preparation these additions amounted to 17 m-mole/l. $CaCl_2$ (added as a 0·5 M solution), 10 m-mole/l. Na_2HPO_4, 1·25 m-mole/l. Na_3 citrate and 10 m-mole/l. NaOH (added as a mixed solution with a phosphate concentration of 0·5 M). The resultant 'milk' was then equilibrated against the corresponding original milk by dialysis. This procedure yielded a 'milk' almost identical with the original milk in colloidal-phosphate content.

(*d*) *Small particle milk.* This was obtained by removing from milk the coarser caseinate micelles by centrifuging it for 30 min in a Sharples separator at 25,000 rev/min. It contained about one-quarter of the original casein of the milk.

The duration of the second stage of coagulation by rennet was determined as described by Pyne & McHenry (1955). Viscosity was determined with Ostwald viscometers (British Standard), and pH by glass electrode. Analytical methods in general were those already used (Pyne & McGann, 1960); others are described as they occur. All analytical values were corrected for any incidental dilution.

RESULTS

(1) *Effect of colloidal phosphate on the formaldehyde titration of milk*

Since the conclusions originally drawn from the study of this effect have recently been challenged this matter will be discussed first.

The fact that adding oxalate to milk increases its formaldehyde titration had been interpreted by Pyne (1934) as being due to the rupture of a chemical link or links (which were presumed to take the form of shared calcium atoms) between the caseinate and the colloidal phosphate of the milk. Evenhuis & de Vries (1956*a*) rejected this explanation, however, and ascribed the relatively smaller formaldehyde titration of unoxalated milk to slow precipitation of calcium phosphate during the preliminary neutralization of the milk and slight solubilization of this material on subsequent addition of formaldehyde. They further noted that the difference between the two titrations diminished on allowing the formaldehyde-treated milks to stand.

To eliminate the postulated effect of precipitation of calcium phosphate on the formaldehyde titration the experiments were repeated using soluble phosphate-low milk and the results compared with those obtained with normal milk and colloidal phosphate-free milk. In a typical experiment addition of oxalate was found to increase the formaldehyde titration of the corresponding milk, soluble phosphate-

low milk and colloidal phosphate-free milk by 8·2, 7·5 and 1·7 % respectively. These results show that precipitation of soluble phosphate had only a slight effect on the differences obtained.

To eliminate the possible solvent action of formaldehyde, whether due to the presence of contaminating acid or to the acidity developed on interaction with the milk proteins, the experiments were repeated, using neutralized formaldehyde to which 90 % of the alkali required to complete the titration had been added beforehand. This modification also was found to have no appreciable effect on the results obtained. Presence of colloidal phosphate is thus confirmed as the factor mainly responsible for the differing formaldehyde titrations of oxalated and unoxalated milk.

A gradual narrowing of this titration difference with time of standing does, on the other hand, seem to occur, as Evenhuis & de Vries (1956*a*) reported. It is accompanied, however, by a considerable increase in both titrations. Thus in a typical experiment the formaldehyde values of unoxalated and oxalated milk were found after 2 h standing to have increased by 10·5 and 6·4 % of their initial values respectively. Continuing interaction between formaldehyde and less reactive groups of the protein than those initially involved are no doubt responsible.

Though colloidal phosphate apparently influences the formaldehyde titration it is admittedly less certain that this constitutes evidence of a caseinate-colloidal phosphate link. But the phenomenon suggests at least, as ter Horst (1947) pointed out, that the presence of colloidal phosphate in milk can to some degree restrict the accessibility to formaldehyde of certain active groups of the casein.

(2) *Effect of colloidal phosphate on the potentiometric titration of calcium caseinate*

If rupture of calcium caseinate-calcium phosphate links by oxalate with consequent release of further titratable groups is capable of producing the increase in the formaldehyde titration which accompanies the oxalation of milk, it might reasonably be expected to produce also a change in the buffer capacity of the casein which could be recognized without the necessity of adding formaldehyde. Early work with caseinate-calcium phosphate complexes had in fact pointed to the existence of just such an effect (Pyne, 1934). This finding has now been confirmed in experiments made on more strictly comparable systems, namely milk and the corresponding colloidal phosphate-free milk, both largely depleted of soluble phosphate by identical dialysis treatments. Fig. 1 illustrates the course of the potentiometric titrations of these two systems with NaOH. A 3 min interval was allowed after each addition of alkali before reading the pH.

A distinctly lower base-binding capacity of the caseinate is seen to obtain in the presence of colloidal phosphate, even though the milk had lost about one-tenth of its colloidal phosphate during the dialysis employed to free it from soluble phosphate, and probably retained in so doing slightly more soluble phosphate (i.e. more buffer material) than the corresponding colloidal phosphate-free milk. It is noteworthy, moreover, that the titration difference occurs in the pH range where phosphate ester groups are most likely to contribute to the acidity of the protein (Eilers, 1947).

These titration differences resemble those of the corresponding formaldehyde titrations in exhibiting a tendency to diminish with time of standing.

It is recognized that neither this result nor the formaldehyde titration results point unequivocally to the existence of a caseinate-colloidal phosphate link. Both results, considered in isolation, may in fact merely reflect the influence of different degrees of caseinate dispersion on the accessibility of relevant groups. However, in the context of other phenomena associated with the presence of colloidal phosphate to be discussed later these results assume more significance.

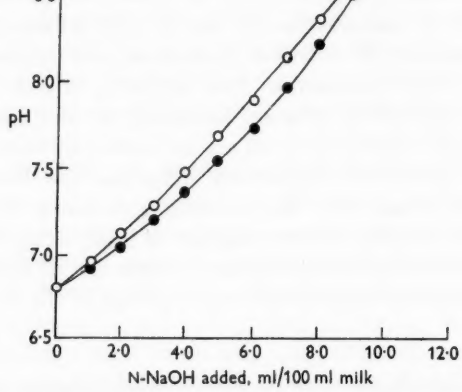

Fig. 1. Potentiometric titration of milk (○—○) and corresponding colloidal phosphate-free milk (●—●) with sodium hydroxide.

(3) *Contribution of colloidal phosphate to the viscosity of milk*

Colloidal phosphate-free milk is strikingly translucent compared with ordinary milk and clearly its caseinate is in a more highly dispersed and perhaps even structurally different condition. Removal of colloidal phosphate was thought likely, therefore, to bring about a change in the viscosity of milk, and this proved to be so. Fig. 2 shows how variation in the colloidal phosphate content affects the viscosity of milk. All viscosities are recorded in terms of the original viscosity of the unaltered milk, which for convenience is taken as 100. The average viscosity of the unaltered milk was about 1·7 cP at 20 °C. Progressive removal of colloidal phosphate leads to a steady increase in the viscosity of milk. When removal of phosphate is complete the viscosity reaches a value about 30 % higher than that of the original milk. Structural alterations in the caseinate micelles caused by removal of colloidal phosphate probably include not only changes of size and shape but also changes in the degree of interaction of the various casein components contained therein, as well as increased availability of certain polar groups and increased hydration. A striking result of the changes which accompany removal of colloidal phosphate is, as will be seen, the greatly increased sensitivity to calcium ions acquired by the caseinate.

Increases in colloidal phosphate content on the other hand, while increasing the opacity of the milk, do not if moderate affect its viscosity greatly (Fig. 2). This result suggests that differences in the degree of caseinate dispersion arising from difference in colloidal phosphate content are in themselves less important in determining the viscosity of milk than are the structural changes in the caseinate micelle caused by withdrawal of colloidal phosphate. The increase in viscosity which finally sets in when the calcium phosphate content of milk is brought to a relatively high value—also shown in Fig. 2—is probably of different origin from that caused by withdrawal of colloidal phosphate and is perhaps due to incipient co-precipitation of caseinate and calcium phosphate (cf. action of calcium saccharate on milk (Pyne, 1929)).

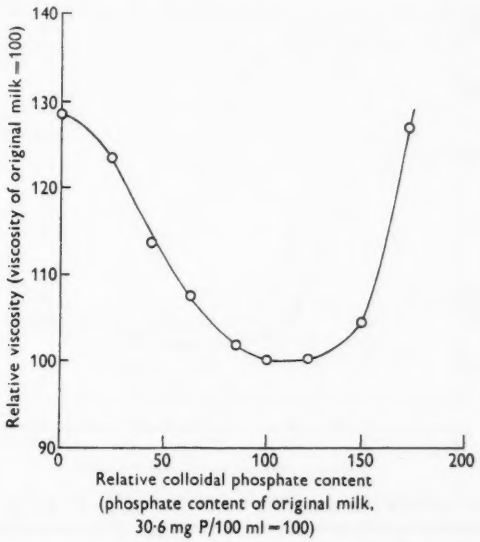

Fig. 2. Effect of altering the colloidal phosphate content of milk on the viscosity of the milk.

(4) *Effect of colloidal phosphate on the stability of milk to calcium ions*

Normal milk is extraordinarily stable to calcium ions. It shows, for instance, no sign of coagulation when calcium chloride is added to give concentrations of up to 1 mole/l. at temperatures from 0 to 40 °C, provided the pH is not subsequently readjusted to its initial value—a procedure which leads to the precipitation of much calcium phosphate and partial co-precipitation of caseinate. Colloidal phosphate-free milk, on the other hand, is relatively unstable to calcium ions, coagulating readily when the concentration of calcium chloride added under similar conditions is only 25 m-mole/l.

To study the effects of removal of intermediate amounts of colloidal phosphate on stability it was desirable first to establish the optimum calcium-ion concentration for milk coagulation in general, and then to employ this optimum in conjunction with a coagulation technique applicable to the whole range of systems examined. Partial rennetting was found to provide both a means of fixing the value for optimum coagulation requirements and the necessary technique for utilizing it. The optimum

calcium concentration for coagulation of milk casein was taken to be that concentration of calcium chloride which, when introduced into normal rennet-treated milk, coagulated it in the minimum time after addition of rennet. (Coagulation was attained by the addition of 2 % of a rennet extract so diluted as to give a minimum clotting time of 6–16 min.) It was found to lie in the region of 25–50 m-mole/l. $CaCl_2$ at 25 °C or over (Fig. 3), which is in fair agreement with the optimum calcium salt concentration for aggregation of caseinate obtained by Dyachenko (1959) using light-scattering methods.

Using a similar rennetting technique the relative stabilities to calcium of milks, varying in colloidal phosphate content from 0 to 170 % of the original value, were

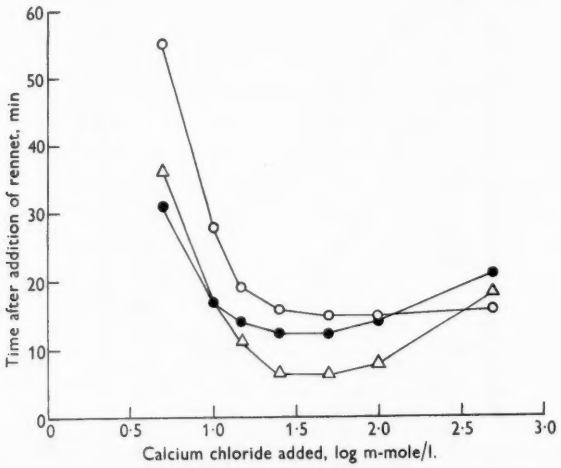

Fig. 3. Time after addition of rennet at 15 °C (○—○), 25 °C (●—●), and 40 °C (△—△) needed to ensure instant coagulation on subsequently bringing the rennet-treated milk to various calcium chloride concentrations.

likewise determined. The results are expressed in terms of the minimum rennetting time at 26 °C required by each milk to form a coagulum under comparable rennetting and coagulation conditions (i.e. 2 % of a commercial rennet extract diluted 100-fold; coagulation within 15 sec after addition of an equal volume of a solution containing 50 m-mole/l. $CaCl_2$). The scale of coagulabilities thus obtained is of course an arbitrary one, but it serves to demonstrate how the stability of milk to calcium salts depends on the concentration of colloidal phosphate present. Typical results (Fig. 4) illustrate this dependence; stability falls steadily with progressive removal of colloidal phosphate, and is low enough to allow coagulation by the calcium chloride solution employed, without previous rennet treatment, when removal of colloidal phosphate exceeds about 50 %. Increasing the colloidal phosphate content up to some 50 % of that originally present is seen, on the other hand, to have little effect on the stability of the milk. Increased sensitivity to calcium only appears when the calcium phosphate content is raised much above the normal milk level. It is significant that sensitivity to calcium, as shown by these results, runs somewhat parallel

to the changes in the viscosity of the milk simultaneously induced by alteration of its colloidal phosphate content.

These stability results and the preceding viscosity observations suggest that colloidal calcium phosphate is a factor in the remarkable stability to calcium of the natural caseinate micelle of milk, and is in some degree responsible for the characteristic internal structure of these micelles. Colloidal phosphate may also provide an integrating factor which serves to preserve the micelle against disruption. It is noteworthy, in this connexion, that fractionation of caseinate for the isolation of κ-casein is normally preceded by disintegration of the natural milk micelle by reagents such as oxalate and citrate (Waugh & von Hippel, 1956; Waugh, 1958) which effectively remove colloidal phosphate and calcium from caseinate.

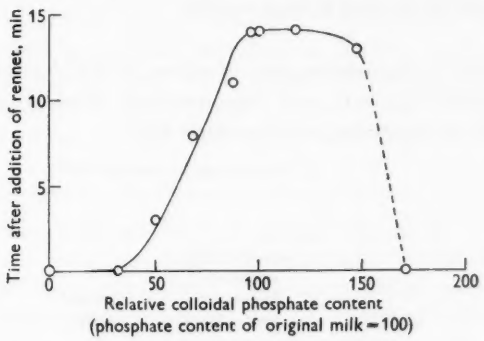

Fig. 4. Effect of altering the colloidal phosphate content of milk on the time after addition of rennet needed to ensure instant coagulation on the addition of an equal volume of a solution containing 50 m-mole/l. $CaCl_2$.

That removal of colloidal phosphate is at least a condition of the fractionation of caseinate by calcium chloride seems to be shown by the following experiment. Normal milk and colloidal phosphate-free milk, both freed from soluble phosphate by previous dialysis, were treated by the Waugh & von Hippel (1956) procedure for the splitting of first cycle casein (i.e. cooling to about 0 °C, addition of 0·25 M-$CaCl_2$ and readjustment of the pH to 6·8, followed by warming to 37 °C and standing overnight). Normal milk treated in this way remained quite stable and unchanged. On the other hand colloidal phosphate-free milk, which is translucent, became quite opaque and gradually formed, like first cycle casein, a coarse precipitate, leaving a clear supernatant liquid in which a coagulum formed rapidly on the addition of rennet, presumably corresponding to fraction S of Waugh & von Hippel. Similar results were also obtained when addition of calcium chloride and adjustment of the pH were made at 37 °C, a procedure which Waugh (1958) found to give rise to stable micelles when applied to first cycle casein. These results suggest that removal of κ-casein from the caseinate micelle by treatment with calcium chloride is prevented, in the natural milk micelle, by the presence of colloidal phosphate.

Note. (*a*) The acidification treatment used in the first stages of the preparation of colloidal phosphate-free milk is not in itself responsible for either the increased viscosity of the milk or its increased sensitivity to calcium. Colloidal phosphate-

low milk (colloidal phosphate content reduced by two-thirds) prepared, at approximately constant pH and calcium-ion concentration, by prolonged dialysis of milk against an artificial phosphate-free milk serum (containing 3 m-mole/l. $CaCl_2$ at pH 6·8) shows much the same properties.

(*b*) Differences in rate of rennin attack also play no part in the stability differences found to accompany variations in the colloidal phosphate content of milk (using the partial rennetting technique described). The caseinate of milk and of the corresponding colloidal phosphate-free milk appear to undergo equally rapid rennin attack as indicated by their practically identical rates of release of non-protein nitrogen (NPN) soluble in 12 % trichloroacetic acid (TCA) under similar conditions (Table 1). The NPN values shown were estimated by the colorimetric method of Lowry, Rosenbrough, Farr & Randall (1951); estimations of NPN made in another instance by the Kjeldahl method yielded similar results.

Table 1. *Comparisons of the relative rates of release of NPN soluble in* 12 % *TCA on treatment with rennet:* (1) *milk and corresponding colloidal phosphate-free milk;* (2) *diluted milk and corresponding small particle milk*

	Percentage increase in NPN			
	(1)		(2)	
Time of rennet action, min	Milk	Corresponding colloidal phosphate-free milk	Diluted milk	Corresponding small particle milk
7·5	29·8	28·9	—	—
10	—	—	8·6	11·6
15	49·2	51·0	—	—
20	—	—	11·8	—
30	70·0	71·6	—	—
40	—	—	14·8	17·8
45	75·2	77·0	—	—
60	—	—	17·8	22·8

(5) *Effect of colloidal phosphate on the second stage of rennet action*

The presence of colloidal phosphate has just been shown to have no influence on the first or enzymic stage of rennet action; it is known, however, to have a considerable destabilizing influence on rennet-altered caseinate, allowing it to coagulate at lower calcium-ion concentrations than would otherwise be possible (Pyne, 1953). Colloidal phosphate-free milk will not, for instance, coagulate with rennet under the conditions which normally lead to coagulation in the corresponding normal milk.

This behaviour contrasts, at first sight, with the stability results obtained for milk and colloidal phosphate-free milk where, as has been seen, the position is the reverse, colloidal phosphate-free milk being much the more calcium-sensitive of the two. Difference in micellar size (probably critical in the second stage of rennet coagulation) may explain this apparent anomaly. The greater stability to calcium of the relatively coarse caseinate-phosphate micelles of normal milk may in part be due to a higher surface concentration of κ-casein. Removal or disintegration of this component by

rennet action could lead, if this were so, to the formation of larger paracaseinate-calcium phosphate micelles whose very size might facilitate their rapid aggregation at the relatively low calcium-ion concentration in ordinary milk; the converse could apply to the much more highly dispersed caseinate of the colloidal phosphate-free milk. However, structural factors probably operate here too, as will appear on considering later the behaviour of synthetic milk.

(6) *Effect of colloidal phosphate on the heat coagulation of milk*

The heat coagulation of milk is a complex phenomenon involving many factors, of which colloidal phosphate content is a relatively minor one. Nevertheless, alterations in the colloidal phosphate content of milk have been shown to influence appreciably the tendency of milk to coagulate on heating, an increase in this constituent leading to a fall in coagulation time and conversely (Pyne, 1958). The effect somewhat resembles that exercised by colloidal phosphate on the second stage of rennet coagulation and may similarly be ascribed, in part at least, to the influence of this phosphate on the size of the caseinate micelles. Micellar structure does not seem to be involved here, however. Thus almost identical heat coagulation times are obtained whether the colloidal phosphate is that originally present in the milk or is synthetic material subsequently introduced into it (Table 3). This result, which does not hold for other properties of milk to be discussed later, is perhaps due to heat-induced changes in the physical condition and dispersion of the colloidal phosphate similar to those described by Evenhuis & de Vries (1956*b*). Such changes might perhaps have the result of rapidly bringing all pre-existing types of colloidal phosphate to a common condition, so that only quantitative differences remained operative.

(7) *Effect of colloidal phosphate on the coagulation of milk by ethanol*

Coagulation by ethanol contrasts strongly with the coagulations so far studied in being almost entirely unaffected by variations in the colloidal phosphate content of milk. Thus a milk which required an equal volume or more of 80–82% (v/v) ethanol retained exactly the same sensitivity to ethanol when its colloidal phosphate content was reduced by 100% or increased by 70%. This result supports the findings of Davies & White (1958) who noted that the concentration of calcium ions in milk is the most important factor controlling the stability of the caseinate micelles to ethanol.

Ethanol presumably acts by reducing the dissociation of the carboxylate groups of calcium caseinate.

(8) *Effect of colloidal phosphate on the acidity developed in milk on addition of calcium salts*

Addition of neutral calcium salts to milk has long been known to cause a lowering of pH. This effect arises partly from the action of these salts on caseinate, and is attributed by Hostettler & Rüegger (1950) to replacement of zwitterion protons by calcium. Lowering of pH is also due partly to changes in phosphate equilibrium, as is clear from the marked pH fall produced by the addition of calcium salts to whey.

To determine whether the presence of colloidal phosphate plays any part in this phenomenon, samples of milk and colloidal phosphate-free milk, both largely freed from soluble phosphate by previous dialysis against an artificial phosphate-free serum, were titrated potentiometrically with neutral M-$CaCl_2$. The results (not shown) indicated that the presence of colloidal phosphate plays no part in the development of the acidity produced.

The phenomenon is probably related to that of stability to ethanol, just discussed. Reduction in the dissociation of calcium caseinate can clearly be attained equally by an increase in the concentration of calcium ions, as here, or by addition of ethanol. No doubt the same groups (presumably carboxylate) are involved in both phenomena, so that colloidal phosphate—which if linked to casein is more probably attached at phosphate centres (cf. §2)—need play no part in either.

The shift in pH towards the acid side produced by adding about 120 m-mole/l. $CaCl_2$ to soluble phosphate-free milk averages about 0·4; that produced in normal milk has been found to be about 0·9. The additional shift clearly comes from changes in phosphate equilibrium.

(9) *Effect of colloidal phosphate on the retention of β-caseinate in the caseinate micelle*

When calcium caseinate solutions are coagulated by rennet below 20 °C much or all of the β-caseinate remains in solution (Pyne, 1951). It was of interest to examine how far this behaviour was modified when the caseinate rennetted was that of normal milk. Accordingly, milk and colloidal phosphate-free milk, both largely freed from soluble phosphate, were cooled to 0–2 °C and treated with an appropriate amount of rennet extract. In order to ensure proper coagulation at 0–2 °C both systems were raised to a calcium chloride concentration of approximately 25 m-mole/l. by the addition of M-$CaCl_2$ before adding the enzyme. The coagulated milks were allowed to stand for some hours and filtered cold. The β-caseinate contents of the filtrates were then estimated. For this purpose β-caseinate was taken to be the protein which separated on warming the filtrates to 30 °C and which redissolved again on cooling. Estimations were made by the amido-black method of Schober & Hetzel (1957) and are only approximate.

In two such experiments the β-caseinate remaining in solution after the coagulation of milk and colloidal phosphate-free milk amounted to 0·39, 0·29 and 0·99 and 0·89 % respectively, indicating that much more β-caseinate was co-precipitated with the α-paracaseinate in the presence of the normal colloidal phosphate of milk than in its absence.

In a modification of this experiment in which the milk and the colloidal phosphate-free milk were coagulated by rennet at 36 °C (instead of at 0–2 °C), then cooled to 0–2 °C and filtered cold 12 h later, the corresponding β-caseinate contents of the filtrates were found to be nil and 0·34 % respectively. Co-precipitation of β-caseinate with α-paracaseinate was naturally much greater here for each milk than in the preceding experiment owing to the relative insolubility of calcium β-caseinate above 20 °C (Pyne, 1951), but was nevertheless incomplete in the absence of colloidal phosphate. This substance thus appears to play an integrating role in the association of β-caseinate with the other casein components in the natural-milk casein micelles.

This influence of colloidal phosphate on the integration of β-caseinate into the caseinate micelle may explain why on ultracentrifugation ter Horst (1950), working with calcium caseinate solutions, obtained partial casein fractionation as evidenced by the variation in the protein/organic phosphorus ratio of the material centrifugally separated, while on the other hand Ford, Ramsdell & Landsman (1955) and Ford & Martinez-Mateo (1958), using normal milk, came to the conclusion that almost all the caseinate exists as a single phosphoprotein.

(10) *Effect of colloidal phosphate in casein systems of differing micellar size*

In discussing the relative stabilities of milk and colloidal phosphate-reduced milk to calcium salts, attention has so far been concentrated on the differentiating phosphatic factor to the exclusion of the dispersion factor. Since the degree of dispersion is, however, always different in the two systems it seemed desirable to obtain some estimate of its influence on stability in isolation from other factors. For this purpose the stability to calcium salts of small particle milk (Sharples's centrifugate, containing about 25 % of the original casein) has been compared with that of the corresponding uncentrifuged milk diluted to the equivalent casein concentration with dialysis-neutralized isoelectric milk serum. Included for comparison were also the colloidal phosphate-free systems derived from each of the foregoing milks. Prior to test all four milks were equilibrated against the original milk by dialysis. The small particle milk and the two colloidal phosphate-free milks, unlike the diluted uncentrifuged milk, appeared almost whey-clear and obviously contained their

Table 2. *Viscosity and coagulability of small particle milk, correspondingly diluted uncentrifuged milk, and their respective colloidal phosphate-free milks*

	Colloidal phosphate content, mg P/100 ml	Relative viscosity at 11·6 °C	Time after addition of rennet for instant coagulation on addition (1:1) of 50 m-mole/l. $CaCl_2$, min
Diluted milk	7·42	100·0	14
Small particle milk	5·24	100·0	14
Diluted milk (colloidal phosphate-free)	—	104·4	2
Small particle milk (colloidal phosphate-free)	—	102·7	6

casein in a highly dispersed condition. The data for the milks, including their stability values (determined by the rennetting technique already described), are set out in Table 2. The findings may be summarized as follows:

(*a*) Small particle milk has a lower content of colloidal phosphate than the corresponding unfractionated milk, in agreement with the results of others (Hostettler & Rychener, 1949; Ford *et al.* 1955). On removal of colloidal phosphate it suffers a correspondingly smaller change of viscosity than the diluted milk.

(*b*) Small particle milk has a stability to 50 m-mole/l. $CaCl_2$ approximately equal to that of the correspondingly diluted uncentrifuged milk, but it becomes the more stable of the two on removal of colloidal phosphate from both.

On withdrawal of colloidal phosphate, small and normal particle milks thus appear to undergo qualitatively similar but quantitatively different alterations in micellar structure, as evidenced by their changes in viscosity. The change in viscosity so

induced is for each milk, moreover, roughly proportional to the initial colloidal phosphate content of the milk.

The superior stability of the colloidal phosphate-free milk prepared from small particle milk compared with that prepared from mixed particle milk suggested that the former might be the richer in κ-casein. The correctness of this supposition was confirmed on determining the amounts of NPN soluble in 12% TCA released from both milks on treatment with rennet under identical conditions. Small particle milk was found to yield much more of this material than the original equivalently diluted milk (Table 1). A similar conclusion, reached by Sullivan, Fitzpatrick & Stanton (1959) from the estimation of the *N*-acetylneuraminic acid content of the casein fractions obtained by the differential centrifuging of milk, came to our notice only after completion of this work.

The somewhat similar stabilities to 50 m-mole/l. $CaCl_2$ of small and mixed particle milks (as determined by the rennetting technique used), despite the greater κ-casein content of the former, seemed surprising. It results, perhaps, from a fortuitous balance of opposing factors. The micelles of small particle milk, though possessing a relatively high κ-casein content, may nevertheless have a lower surface concentration of this constituent than the generally larger micelles of the mixed particle milk. Both systems might thus happen to attain somewhat similar degrees of sensitivity to 50 m-mole/l. $CaCl_2$ in the same time after inception of rennet action while continuing, naturally, to differ in the proportion of κ-casein which had undergone disintegration in each.

An additional, or perhaps an alternative, factor in the similar stabilities of small and mixed particle milks just discussed may be the stabilizing influence of colloidal phosphate itself. It is possible that the colloidal phosphate present in the natural milk micelle possesses independent stabilizing properties supplementary to those of κ-casein—a view consistent with its influence on the viscosity of milk, on the stability of milk to calcium salts and on the degree of integration of the casein fractions within the micelle.

Moreover, the two constituents appear to vary inversely, colloidal phosphate being highest and κ-casein lowest in the larger micelles and conversely for small micelles. Variation in colloidal phosphate content may be the basis of the remarkable polydispersion of the casein in milk, and the inverse phosphate/κ-casein relation observed a natural consequence of the accompanying surface/volume variation.

(11) *Effect of re-introducing colloidal phosphate into colloidal phosphate-free milk*

The presence of colloidal phosphate in milk has been seen to influence many of the properties of the casein. It was of interest to determine whether colloidal phosphate other than that originally present in milk could produce the same effects. Reintroduction of colloidal phosphate into colloidal phosphate-free milk was therefore effected and the synthetic product compared as regards its viscosity, coagulability and other properties with both the original milk and the colloidal phosphate-free material. The synthetic milk had a colloidal phosphate content almost identical with that of the original milk, but was somewhat less opaque. Its relevant properties, and, for comparison, those of the equivalently diluted original and colloidal phosphate-

free milks, are set out in Table 3. As usual all three milks were equilibrated by dialysis against the original milk before being examined.

Re-introduction of the colloidal phosphate is seen to restore to very different degrees the various properties of the original milk. The various results of this treatment—each accompanied by a brief comment—are as follows:

(*a*) A definite increase in opacity, but no corresponding change in viscosity: the degree of dispersion is apparently reduced but the structure of the original milk micelles is not restored, as indicated by the unchanged viscosity.

Table 3. *Comparison of normal milk, synthetic milk and colloidal phosphate-free milk in regard to certain properties*

	Relative viscosity at 19·0 °C	Time after addition of rennet for instant coagulation on addition (1:1) of 50 m-mole/l. $CaCl_2$, min	Duration of second stage of rennet action at 26·2° C, sec	Heat coagulation time at 130 °C, min	Ethanol (%, v/v) required for coagulation (1:1 addition)	Percentage β-casein in rennet filtrate at 0 °C
Original milk	100·0	17	52	55	74–76	0·12
Synthetic milk	120·0	5	35	56	74–76	0·18
Colloidal phosphate-free milk	120·0	0	Did not coagulate	86	74–76	0·35

(*b*) A moderate increase in stability to calcium, which, however, is far from attaining that of the original milk: colloidal phosphate appears to have some stabilizing effect even where, as here, it is not an original constituent of the milk.

(*c*) A considerably increased sensitivity to the second or calcium precipitation stage of rennet coagulation which now exceeds even that of the original milk: this is due presumably to the reduced dispersion of this initially calcium-sensitive but highly dispersed system (cf. §5).

(*d*) Increased sensitivity to heat, which is now restored to about the same level as in the original milk. Heat coagulation appears to be independent of micellar structure, as already suggested (cf. §6).

(*e*) No effect on sensitivity to ethanol: an expected result in the light of earlier observations (cf. §7).

(*f*) An increased retention of β-casein by the rennet coagulum, falling short however of the retention of β-casein found for the original milk: solubility of β-casein is perhaps reduced in presence of calcium phosphate. This point is under investigation.

The various results enumerated are, generally speaking, in accordance with the evidence already presented and the interpretation given to it. Apparently there is a special structure of the natural casein micelle to which the colloidal phosphate contributes. Removal of this constituent brings about changes in the properties of the casein micelles and thereby of the milk, which cannot be fully reversed by subsequent re-introduction of colloidal phosphate.

DISCUSSION

Alterations in the colloidal phosphate content of milk have been shown to affect markedly certain properties of the milk to an extent which varies considerably depending on whether the phosphate was present initially or was subsequently introduced.

Thus removal of colloidal phosphate brings about a considerable increase in the viscosity of the milk and in its sensitivity to calcium, and causes an apparent reduction in the degree of association of the various casein components. The resulting milk, for instance, is less capable than before of retaining β-casein in the rennet clot formed at low temperatures, and is no longer resistant to fractionation by calcium salts. Re-introduction of colloidal phosphate into milk which has been freed from this constituent reverses only partially the effects of its previous removal. It exercises, for instance, no appreciable effect on the viscosity of the 'milk' which continues to exceed greatly that of the original milk, presumably because it fails also to restore the original micellar condition of the casein. Re-introduction of colloidal phosphate does, however, restore to some degree the stability of the milk to calcium salts; it also improves considerably the retention of β-casein in the rennet clot.

Rennet-altered milk provides at first sight an apparent exception to the stabilizing action of colloidal phosphate since it coagulates more readily than the corresponding rennetted colloidal phosphate-free milk. But this behaviour appears, as earlier discussed, to be due mainly to differences in the degree of dispersion of the two milks, which is critical for coagulability at the relatively low calcium-ion concentrations operating.

Colloidal phosphate also destabilizes milk somewhat to coagulation by heat, but the whole phenomenon, which involves, *inter alia*, considerable changes in phosphate equilibrium (Evenhuis & de Vries, 1956 *b*) and in the physical condition of the colloidal phosphate, is too complex for this result to be quite relevant to the present discussion.

It is difficult to avoid the conclusion that colloidal phosphate has some specific integrating and stabilizing function as a component of the natural micelles of milk casein. The fact that micellar size is directly related to colloidal phosphate content and inversely to κ-casein content suggests that the colloidal phosphate content may be a factor of importance in determining the size of the caseinate particles, while the κ-casein content is more a function of their surface area. The marked polydispersion of the natural casein of milk, contrasting as it does with the restricted range of dispersion of calcium caseinate solutions (Philpot, 1938) and, visibly, with that of colloidal phosphate-free milk, witnesses to a certain degree of dimensional and perhaps structural persistence associated apparently with the presence of colloidal phosphate. Variation in the κ-casein content of the calcium caseinate particles of milk is in itself, no doubt, also a factor in their range of dispersion, and probably accounts in part for the phenomenon of 'size memory' described by Choate, Heckman & Ford (1959).

The effects of colloidal phosphate on the behaviour of calcium caseinate as it occurs in milk appear to indicate the existence of an intimate association, probably some type of chemical union, between the two. In considering likely points of attachment the phosphatic groups of the casein components are an obvious choice, and one for which there is some indirect experimental evidence. Both the formaldehyde

and the potentiometric titrations of milk and of colloidal phosphate-free milk furnish evidence—once more confirmed—of some reduction in the accessibility of polar groups of the casein in the presence of colloidal phosphate. This effect is most marked, as the potentiometric titration results show, over the pH range in which the phosphatic groups of the protein contribute strongly to its acidity. The practically unchanged sensitivity of calcium caseinate solution to ethanol, on the other hand, and the equally unchanged degree of exchange of caseinate protons against calcium ions, irrespective of whether colloidal phosphate is present or not, would seem to exclude the carboxylate groups of the protein from participation in the postulated colloidal phosphate-caseinate linkage.

As to possible modes of action of colloidal phosphate as a stabilizing factor in milk, two alternatives suggest themselves. Colloidal phosphate may act as an independent integrating agency in the casein micelle, supplementary in some respects to κ-casein, operating perhaps to cross-link some of the phosphatic groups of the various casein components and thereby reducing the number available for inter-micellar linkage. Or it may act as a 'filler' which by primarily influencing the size and form of the micelles affects also their stability to calcium, but only indirectly and in consequence of the particular surface concentration of κ-casein which these dimensional factors dictate. While the two views are not necessarily mutually exclusive, the former seems to accord better with certain experimental results already noted, e.g. with the apparent impossibility of removing κ-casein from the casein micelles of natural milk by treatment with calcium salts, with the relatively greater retention of β-casein in the rennet curd of such milk, and with the slight but definite stabilizing effect of colloidal phosphate even where it is subsequently introduced into colloidal phosphate-free milk.

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The effect of feed supplements on the yield and composition of milk from cows grazing good pasture

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Summary. Two experiments are described in which spring-calved Ayrshire cows were fed supplementary food when grazing good, leafy pasture.

In the first experiment 6 lb of a mixture of three parts bruised oats and one part flaked maize was fed daily to three groups of five cows. One group received the supplement from 1 July until 30 October, and the other two groups from 1 August and 1 September respectively. A control group received no supplement.

In no month was there any statistical difference in daily milk yield between supplemented and unsupplemented groups of cows. The group fed from 1 July gave the highest total milk yield in the period June to October, but with a return of only 1 gal of milk from 34 lb of meal.

The length of the lactation was not significantly affected by the treatment.

In the second experiment, three groups of four cows on a 9-week change-over trial (4 May–5 July) grazed herbage containing an average of only 17·6 % dry matter and 3·8 % crude fibre. On the three treatments the cows ate pasture only, pasture plus 1·6 lb hay/day and pasture plus 5·7 lb of meal/day. The meal consisted of four parts by weight of undecorticated cotton cake, four of bruised oats and three of locust beans.

Milk yield was significantly increased from 41·8 lb on the all-grass ration to 43·2 lb/day on the meal treatment but the hay did not affect milk yield.

The fat content of the milk was significantly increased from 3·77 to 3·91 % on the hay treatment, whereas the solids-not-fat content of the milk was not significantly affected by any of the treatments.

At the present-day prices of the feeds and with cows on good leafy pasture it was clearly uneconomic to feed the meal supplements.

The increased emphasis on pasture as a cheap source of food for livestock has been one of the major trends in British agriculture in recent years. Efficient grassland management can ensure an adequate supply of pasture capable of sustaining high-yielding cows (Hamilton, 1952; Holmes, 1954), yet a survey of about 1000 milk recorded herds in England and Wales in 1957, 1958 and 1959 showed that over 90 % of the herds were being fed supplementary concentrates during the last week in May when the pastures were at about their best (Milk Marketing Board, 1958–59).

Many investigations of the effect of feeding supplements to milking cows at pasture have been undertaken (Corbett & Boyne, 1958; 't Hart, 1956; MacLusky, 1955;

Holmes, 1958). The results of these experiments and many others have been summarized by Corbett (1959), and from them he concluded that in general the response to supplementary feeding in terms of milk yield was small. At best, 4 lb of supplementary concentrates produced only an extra 2–3 lb of milk, and in most of the trials they produced less than half this amount.

From these results and at present feed and milk prices, supplementary feeding at pasture is, therefore, grossly uneconomic. But two points arise from these trials. First, the majority of the trials have been on a short-term basis with feeding periods ranging from 1 to 10 weeks. The importance of the initial phase of lactation on the subsequent length and total lactation yield has been stressed by Blaxter (1956), and it is also known that the response to extra feeding varies according to the stage of lactation (Blaxter, 1959). Secondly, the types of supplements used have generally supplied either additional energy or protein to the ration, whereas Frens (1955) has emphasized the importance of fibre in the diet of cows grazing high-quality pasture which has been intensively fertilized.

To investigate these two aspects of supplementary feeding to grazing cows two experiments were conducted. In 1958 a study was made of the effect of feeding a low-protein, high-energy supplement to grazing cows at different stages of their lactation. In the second experiment the effect of feeding two fibrous supplements was studied during May and June, 1959.

EXPERIMENT 1—1958

Experimental

Animals and experimental design

Twenty Ayrshire cows which had calved between 4 February and 1 May 1958 were used. They were in their second to eighth lactations, and were all on a constant diet from the time of first going to grass in the spring until the experiment started. The twenty cows were divided into five groups of four animals according to their daily milk yield in May, date of calving, and live weight. One cow from each group was then allocated at random to one of four continuous experimental treatments. The experiment lasted 5 months, from 1 June to 30 October 1958.

Feeding

All the cows grazed together as one herd both day and night and all had an equal opportunity to consume herbage of a similar quality. The period 1 to 30 June was considered as a preliminary period and the first experimental feeding did not begin until 1 July. The four experimental treatments were: (A) no supplement (control); (B) 6 lb supplementary meal/day from 1 July to 30 October; (C) 6 lb supplementary meal/day from 1 August to 30 October; (D) 6 lb supplementary meal/day from 1 September to 30 October. The cows on treatments B, C and D were fed the supplement, therefore, for 4, 3 and 2 months respectively.

The supplementary meal consisted of 3 parts by weight of freshly bruised oats and 1 part of flaked maize and had the following composition: dry-matter content 82·4%, crude-protein and crude-fibre content of dry matter (by analysis) 13·4 and 7·8% respectively, calculated starch equivalent 66, and calculated digestible crude protein 8 (Woodman, 1957). Cows receiving the supplementary food were fed 3 lb

of the mixture at each milking time in individual stalls to enable any food refusals to be measured.

The cows were milked twice a day and their yields recorded at each milking to the nearest ¼ lb. In addition, the cows were weighed on one afternoon each week before milking started.

Herbage sampling

Samples of herbage were taken at least twice a week, from the swards which the cows were grazing. The samples were cut by shears to a level similar to that left by the animals, and the dry-matter content was determined and subsequently analysed for crude protein and crude fibre.

Results

Herbage quality and supplement consumption

A total of 17·0 in. of rain was recorded during the 4-month experimental period from 1 July to 30 October, and there was abundant grazing at all times. Thirteen fields were grazed in this period and the mean dry-matter, crude-protein and crude-fibre contents of the herbage, grouped on a weekly basis, are given in Table 1. In only 3 of the 18 weeks was the dry-matter content of the herbage over 20% and a number of very low values was recorded. For example, the value of 14·2% dry matter in the week which began on 15 July was attributable to a direct reseed which was being grazed. The crude-protein contents were, in general, at a satisfactory level with the lowest value recorded in mid-August.

Table 1. *Experiment* 1, *mean weekly dry-matter, crude-protein and crude-fibre contents of the swards grazed*

Week beginning	Dry-matter content, %	% of dry matter		Week beginning	Dry-matter content, %	% of dry matter	
		Crude protein	Crude fibre			Crude protein	Crude fibre
1 July	16·3	18·6	24·1	2 Sept.	16·8	17·0	23·1
8	16·5	16·8	23·3	9	18·6	15·2	25·0
15	14·2	20·4	21·5	16	16·4	15·3	26·9
22	25·9	19·7	19·4	23	—	—	—
29	20·4	16·6	24·6	30	16·8	17·4	25·1
5 Aug.	18·1	13·2	27·0	7 Oct.	20·1	15·6	26·2
12	14·8	14·3	27·7	14	19·1	18·5	23·0
19	15·9	13·1	27·6	21	18·8	14·8	25·2
26	16·2	15·3	23·7	28	19·6	14·3	29·4

The supplement was palatable and refusals were virtually nil. The total quantity of supplement consumed on each treatment averaged 0, 732, 546, and 360 lb/cow for treatments A, B, C and D respectively.

Milk yield

The mean daily milk yields on each treatment are given in Table 2. It will be seen that in June, the preliminary month when no supplement was fed, the mean yields were similar for all the groups with a range from 40·1 lb/day on treatment D

to 42·0 lb/day on treatment B. From July onwards as the supplements were fed on the different treatments the mean daily yields declined, but in no month was the yield of cows receiving supplements significantly different from that of cows without supplements. In October, the last month of the experiment, the mean yield of the cows on treatment A, on which no supplements had been fed, was 21·4 lb/day and the mean yield on the three treatments B, C, and D, in which supplements were given, was 21·3 lb/day. When the yields for the entire period 1 June to 31 October are compared (Table 2) it will be seen that treatment B gave the highest yield, 4920 lb, compared with the yield of 4700 lb for the control treatment, but that the mean value for treatments B, C and D was the same as that for treatment A, namely, 4700 lb. The mean lactation yield of the twenty cows on the experiment was 8800 lb, with lactation lengths ranging from a mean of 255 days on treatment C to 289 days on treatment D (Table 2). There was no clear cut relationship between treatment and length of lactation.

Table 2. *Experiment* 1, *mean daily milk yield for the months June to October, total yield and length of lactation*

Treatment	Supplement fed after	Mean daily milk yield, lb					Total yield, lb, 1 June–31 Oct. inclusive	Length of lactation, days
		June	July	Aug.	Sept.	Oct.		
A	None fed	41·5	36·6	30·6	24·4	21·4	4700	262
B	1 July	42·0	38·5	34·1	27·6	19·4	4920	274
C	1 Aug.	40·6	32·6	27·4	21·0	21·3	4350	255
D	1 Sept.	40·1	36·7	31·5	27·5	23·2	4840	289

Table 3. *Experiment* 1, *monthly regression coefficient of milk yield on time, lb/day*

Treatment	June	July	August	September	October
A	−0·32	−0·12	−0·04	−0·15	−0·15
B	−0·34	−0·08	−0·03	−0·26	−0·22
C	−0·45	−0·20	−0·11	−0·19	−0·15
D	−0·38	−0·04	−0·08	−0·12	−0·14
Pooled regression	−0·37	−0·11	−0·06	−0·18	−0·17

Regression coefficients of daily milk yield on time were calculated for each month of the experiment and are given in Table 3. There were no statistically significant differences in any month between treatments. The largest decline in yield occurred in June, when the pooled regression was −0·37 lb/day, but thereafter the rates of decline were much less.

Live weight

The linear regression coefficient of live weight on time was calculated for each cow on each treatment every month. With the exception of the cows on treatment A in October there was an increase in live weight on each treatment every month and no significant difference was found between any of the treatments at any time.

EXPERIMENT 2—1959

Experimental

Animals and experimental design

Nine cows which had been on the previous trial in 1958, and three first-lactation heifers were used in this experiment. Ten of the animals had calved in March and the range of calving dates was 8 March to 19 April 1959. The cattle were all on the same constant diet for 4 weeks before the experiment started and were then divided into four groups of three animals on the basis of calving date, yield and age. The three animals in each group were allocated at random to one of three treatment sequences in two sets of complementary 3 × 3 Latin squares (Cochran, Autrey & Cannon, 1941). The experiment lasted 9 weeks from 4 May to 5 July 1959 and was divided into three equal periods. The change from one treatment to another was made in one day and in calculating the results only data from the last 2 weeks in each period were used.

Feeding

As in the previous experiment all the twelve cattle were grazed together as one herd and the three experimental treatments were: (A) no supplement (control); (B) 4 lb of hay/day offered to each cow; (C) 6 lb of supplementary meal of high fibre content/day offered to each cow.

The hay was of complex botanical composition and at the flowering stage. It had been made in 1958 and had the following composition: dry-matter content 85·7 %; crude-protein and crude-fibre contents of dry matter, 7·4 and 37·9 % respectively. The supplementary meal consisted of the following in parts by weight: 4 undecorticated cotton cake, 4 bruised oats and 3 kibbled locust beans. The dry-matter content was 86·0 % and the dry matter contained 17·2 % crude protein and 15·5 % crude fibre. The calculated starch equivalent was 55 and the digestible crude protein 10 %.

The hay and the meal of treatments B and C respectively were offered to the cows twice a day in the byre at milking time and any refusals of food recorded.

The milk was weighed at each milking, and samples were taken from consecutive evening and morning milkings twice a week for the determination of fat and solids-not-fat content. Also, the twelve cattle were weighed on one afternoon per week just before milking. As the animals were to graze young intensively manured pasture they were all fed 1 lb of anti-tetany cubes per day for the 9 weeks of the experiment. This supplied 1 oz of calcined magnesite per cow per day.

In periods 2 and 3 of the experiment samples of faeces for the determination of dry-matter content were collected from the cows immediately after the afternoon milking.

Samples of herbage were taken as in Expt. 1.

Results

Herbage quality and supplement consumption

Grass of good leafy quality was available to the cows throughout the 9-week experimental period, and some details of this are given in Table 4. The dry-matter content of the herbage was low, with a mean value of 17·6 % over the entire experi-

mental period. The crude-fibre content of the herbage dry matter was also low, averaging 21·5 %.

The daily consumption of hay varied both with the individual animal and the period of the experiment. In periods 1, 2 and 3 the daily intake was 1·4, 1·7 and 1·8 lb per cow per day with a mean consumption of 1·6 lb/day for the whole trial. Some refusals of meal occurred in period 1, but in the remainder of the trial it was regularly eaten and the mean intake was 5·7 lb/day.

Table 4. *Experiment 2, mean weekly dry-matter, crude-protein and crude-fibre contents of the swards grazed*

Week commencing	Dry matter, %	Crude protein in dry matter, %	Crude fibre in dry matter, %
4 May	21·0	16·8	18·2
11	15·0	20·3	22·0
18	16·3	16·1	24·2
25	21·2	17·1	22·2
1 June	18·0	14·5	24·3
8	18·2	21·6	19·5
15	18·6	19·7	20·4
22	17·4	20·1	19·6
29	13·0	17·1	23·5
Mean	17·6	18·1	21·5

Table 5. *Experiment 2, mean daily milk yield, fat and solids-not-fat contents of the milk, and live weight*

Treatment	Milk yield/day, lb/cow	Fat, %	Solids-not-fat, %	Live weight, lb
A (control)	41·8	3·77	8·81	1029
B (hay)	41·4	3·91	8·74	1032
C (meal)	43·2	3·76	8·79	1032
S.E. of difference between two means	±0·35	±0·06	±0·05	±3

Milk yield and composition

Details of the average milk yields per cow and the composition of the milk are shown in Table 5. The treatments had no substantial effect on daily milk yield. Cows receiving the hay (treatment B) gave yields which were not significantly different from the yields of the control group, and the increase on treatment C of 1·4 lb milk per day, although statistically significant ($P < 0·05$), was small. Residual effects of the treatments from one period to another were examined by the method of Cochran *et al.* (1941), but nothing of statistical significance was found. The fat content of the milk on treatment B was significantly higher ($P < 0·05$) than that on the other two treatments. The cows on this same treatment gave milk with the lowest content of solids-not-fat but none of the small differences between treatments was significant.

Live weight

The mean live weights of the cows on the three treatments are also given in Table 5. None of the differences was significant.

Faeces dry-matter content

The average dry-matter contents of the faeces collected from the cows on treatments A, B, and C in the last 6 weeks of the experiment were 11·0, 11·3 and 11·9 % respectively, suggesting that the different treatments had very little effect on the physical condition of the faeces.

DISCUSSION

The results of these two experiments clearly showed that although supplementary feeding at pasture gave some increases in milk yield the increases were exceedingly small. The increase in milk yield was equivalent to 1 gal of milk from 34 lb of meal in Expt. 1 (treatment B) and 1 gal of milk from 42 lb of meal in Expt. 2 (treatment C). These increases are similar to those reported by other workers (Corbett & Boyne, 1958; Holmes, 1958). Furthermore, the feeding of the supplements in Expt. 1 had no significant effect on lactation length and there was no marked evidence that the normal decline in yield in the summer months was halted by the experimental feeding. In fact no compensating advantage was detected. The live-weight changes of both supplemented and unsupplemented cows were similar and there was no striking difference in the bodily condition of the animals in the various groups.

The grass throughout both experiments was always at the leafy stage, and as indicated from analytical results (Tables 1 and 4) it had a high protein content and a relatively low crude-fibre content. In Expt. 2, conducted in May and June, the dry-matter content of the herbage averaged only 17·6 %, and was comparable in that respect to the type of herbage which Frens (1955) considers to be so low in dry matter that it passes too rapidly through the alimentary tract of the cow and produces soft faeces with an abnormally high loss of nutrients. The feeding of a meal of high fibre content in this experiment increased milk production slightly, but had only a very small effect on the dry-matter content of the faeces. The addition of hay to the all-grass diet increased the dry-matter content of the faeces by less than 1 % and had no effect on the daily milk yield. The feeding of the hay did, however, significantly increase the fat percentage of the milk by 0·14, and this would appear to be the only advantage from the treatment. This increase in fat percentage is in agreement with the results of McClymont (1950) and Hildebrandt (1958), although the fat percentage of the milk of the cows in the control group fed on grass only was not unsatisfactory at 3·77 (Table 5).

The feeding of the supplements in these two experiments was uneconomic. The results therefore give further evidence that provided sufficient herbage of good leafy quality is available there are no advantages in feeding supplements to milking cows at pasture.

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The effect of the plane of energy nutrition of the cow during the late winter-feeding period on the changes in the solids-not-fat content of milk during the spring-grazing period

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Summary. Three groups of eight Friesian cows, four of high and four of low milk yield, were given during the last month of the winter-feeding period rations that provided respectively low, normal and high planes of energy nutrition, at approximately 75, 100 and 130% of the Woodman standards for starch equivalent (S.E.). They were then all changed to the grazing of good, spring pasture, without supplementary food. The changes with grazing in solids-not-fat (S.N.F.) content and yield of milk were markedly influenced by the previous level of winter feeding. After 15–18 days of grazing the mean changes in S.N.F. percentage were +0·33, +0·11 and −0·12 for the low, normal and high energy groups, and the corresponding changes in milk yield were +7·70, +2·13 and −0·68 lb per cow per day. The changes in S.N.F. reflected mainly changes in protein content, and to these casein, β-lactoglobulin and α-lactalbumin all contributed. Small, differential changes were found in the fat and lactose contents of the milk. The effects of the level of initial milk yield of the cow on the various responses to grazing are recorded.

These observations support the view that when a rise in S.N.F. with spring grazing occurs it probably results from an improvement in the plane of energy nutrition. There appears to be no need to postulate a special effect of any specific herbage constituent.

When cows are transferred from winter feeding to spring grazing the solids-not-fat content of the milk usually rises, and often by 0·3–0·4%, as illustrated by the data of Provan (1956). Since Riddet, Campbell, McDowall & Cox (1941) and Rowland (1946) demonstrated that underfeeding lowers the S.N.F. content of milk, it has been suggested from time to time, e.g. Rowland (1944) and Waite, White & Robertson (1956), that where an increase in S.N.F. occurs with the change to grazing it may be the result of a higher plane of nutrition. Among other possible explanations offered have been that herbage contains oestrogens which promote the secretion of S.N.F. (Bartlett, Folley, Rowland, Curnow & Simpson, 1948)—a theory now discounted (Pope, McNaughton & Jones, 1959)—and that the high soluble carbohydrate content of young herbage (Waite *et al.* 1956) may stimulate fermentation in the rumen. The various suggestions for the cause of the spring rise in S.N.F. were reviewed by Waite, Castle & Watson (1959).

Rook & Rowland (1959) have already reported that cows on a high plane of energy nutrition for the last few weeks of winter feeding showed a very small and transient increase in S.N.F. percentage during grazing, whereas comparable cows previously on a normal plane showed a typical increase. Also, Waite *et al.* (1959) have shown that the milk of cows on a high plane of nutrition for the last month of winter feeding did not change significantly in S.N.F. level when the ration was changed at the same level of S.E. intake to cut spring grass given in the stall, with some concentrates where necessary.

The object of the present investigation was to seek additional information on the responses in milk composition and yield by changing cows to spring pasture from a wider range of winter plane of nutrition than had been explored before, and to note the effect of the initial level of milk yield, since yield is one of the factors determining the plane of nutrition which the grazing animal achieves.

EXPERIMENTAL

Animals and design

Twenty-four Friesian cows which had been in milk from 1 to 8 months, and were in their first to ninth lactations, were divided according to their milk yields into two equal groups, consisting of those producing 37 lb/day or more, and those producing less. Each group was then subdivided into four lots of three cows, the members of each lot being as similar as possible in stage of lactation and current milk yield. Individuals in each lot were then allocated at random to one of three feeding treatments and maintained on these for a period. The resulting six experimental groups of four cows were then all changed to the grazing of the same spring pasture.

Feeding

Before the experimental period the cows were rationed according to yield on hay, grass silage and a farm-mixed concentrate composed of barley 3 parts, bran 1 part, and decorticated groundnut cake 1 part, by weight. The three experimental feeding treatments were: A, a normal-energy ration providing the Woodman (1954) standards of 7 lb S.E./day for the maintenance of an 11 cwt cow, and 2·5 lb S.E./gal of milk; B, a high-energy ration providing an additional 1·25 lb S.E./gal, that is, about 130 % of the standards; and C, a low energy ration providing only 1·25 lb S.E./gal, that is, about 75 % of the standards. In all rations the protein was in excess of the Woodman (1954) standards.

The maintenance rations consisted of 8 lb of medium quality hay and 30 lb of ensiled brewer's grains per cow per day. For production, groups A and C continued to receive the farm-mixed concentrate and group B was given a concentrate composed of 4 parts of this mixture with 3 parts of flaked maize, by weight. The cows were changed gradually to these feeds over a period of 5 days (19–24 March, 1959) and then fed at constant rates for the following 3 weeks. They were then transferred over a period of 3 days to the grazing of good, spring pasture without any additional food. The pasture was strip-grazed but the allocation was intentionally liberal. From 14 to 26 April the cows were grazing a cocksfoot–short rotation ryegrass–white

clover ley, and from then till 2 May, when experimental observations ceased, a meadow fescue–cocksfoot–timothy–white clover ley. Contribution from the clover was small and the grasses first mentioned were the dominant species.

Sampling and methods of analysis of the milk

Samples of milk were taken at every milking for the last 12 days of the experimental winter-feeding period and throughout the grazing period, and weighted composite samples representing 2- or 4-day periods were prepared for each cow. These samples were analysed for fat by the Gerber method (British Standards Institution, 1955) and for total solids gravimetrically (British Standards Institution,1951), S.N.F. being obtained by difference. Certain of the samples were analysed for lactose, by a modification of the method of Hinton & Macara (1927), for total nitrogen, and for nitrogen distribution by the scheme of Aschaffenburg & Drewry (1959).

RESULTS

For each of the six experimental groups, the mean values for milk yield and the fat, S.N.F., protein (total N × 6·38) and lactose contents of the milk for the last 8 days of the winter-feeding period, and the changes during the grazing period, are given in Table 1. The mean values for the various nitrogen fractions for the last 4 days of the winter-feeding period, and the changes by the last grazing test-period, are given in Table 2. The significance of the effects of the level of winter feeding and of milk yield on the changes in the values with grazing was determined by an analysis of variance.

Milk yield

The changes in milk yield with grazing were influenced by the previous plane of energy nutrition. As would be expected, the cows in the 'low-energy' groups showed the largest increases in yield, ranging from 6 to 10 lb per cow per day. Their increases were significantly greater ($P < 0{\cdot}05$) than those of the other groups. In the 'normal-energy' groups, one cow yielding 64 lb/day—the highest yield in the experiment—declined by 11 lb/day, but the other cows in these groups gained an average of 4 lb/day. The 'high-energy' groups showed a small fall in milk yields with grazing. The differences in response between the 'medium-energy' and 'high-energy' groups just failed to reach significance ($P > 0{\cdot}05$).

The level of milk yield did not have a significant effect on the changes in the yields of the groups with grazing.

Fat

The group responses in fat percentage varied with the levels of winter feeding and milk yield and with the type of ley. In the first two grazing test-periods the fat percentage increased more in the 'high-yield' groups than in the 'low-yield' groups ($P < 0{\cdot}05$). The effect of the level of winter feeding was not significant in the first two grazing test-periods but was highly significant ($P < 0{\cdot}01$) in the third test-period.

Fat percentage usually falls with grazing: the small rises observed in this experiment were probably due to the inclusion of only 8 lb of hay/cow in the previous

winter rations. The lower the proportion of roughage in the winter diet, whether as a result of feeding for a higher level of milk yield or at a higher plane of energy, the greater was the increase in fat content during grazing.

Table 1. *Mean values, with standard deviations, for each group of four cows, for milk yield and the fat, solids-not-fat, protein (total nitrogen × 6·38) and lactose (anhydrous) contents of the milk for the last 8 days of winter feeding, and the group mean changes, with standard deviations, during the grazing period*

	Yield, lb/day	Fat, %	Solids-not-fat, %	Lactose, %	Protein, %
NORMAL-ENERGY WINTER RATION					
'Low-yield' animals					
Winter feeding	26·0 ± 5·4	4·00 ± 0·86	8·78 ± 0·32	4·50 ± 0·15	3·40 ± 0·31
Grazing, days 7–10	+3·2 ± 2·8	+0·09 ± 0·35	−0·04 ± 0·08	+0·02 ± 0·07	−0·02 ± 0·06
11–14	+4·3 ± 2·7	+0·27 ± 0·43	+0·08 ± 0·04	+0·01 ± 0·06	+0·04 ± 0·08
15–18	+4·8 ± 3·9	−0·08 ± 0·48	+0·16 ± 0·08	+0·10 ± 0·15	+0·13 ± 0·12
'High-yield' animals					
Winter feeding	44·7 ± 11·5	3·28 ± 0·20	8·63 ± 0·18	4·54 ± 0·10	3·22 ± 0·14
Grazing, days 7–10	−0·5 ± 3·4	+0·50 ± 0·37	−0·08 ± 0·11	−0·08 ± 0·11	+0·01 ± 0·10
11–14	+0·5 ± 6·1	+0·55 ± 0·41	−0·08 ± 0·13	−0·09 ± 0·13	−0·02 ± 0·11
15–18	−0·6 ± 7·8	+0·14 ± 0·12	+0·06 ± 0·06	0·00 ± 0·15	+0·10 ± 0·17
HIGH-ENERGY WINTER RATION					
'Low-yield' animals					
Winter feeding	31·1 ± 7·9	3·36 ± 0·08	8·68 ± 0·31	4·55 ± 0·18	3·22 ± 0·10
Grazing, days 7–10	−1·6 ± 1·7	+0·52 ± 0·20	−0·12 ± 0·18	+0·01 ± 0·06	−0·05 ± 0·03
11–14	−0·3 ± 3·0	+0·49 ± 0·27	−0·12 ± 0·15	−0·09 ± 0·13	−0·06 ± 0·03
15–18	+0·1 ± 2·5	+0·33 ± 0·18	−0·08 ± 0·08	+0·04 ± 0·07	−0·05 ± 0·05
'High-yield' animals					
Winter feeding	46·7 ± 4·8	3·43 ± 0·20	8·92 ± 0·41	4·62 ± 0·20	3·32 ± 0·21
Grazing, days 7–10	−1·7 ± 4·8	+0·65 ± 0·14	−0·26 ± 0·18	−0·09 ± 0·13	−0·05 ± 0·10
11–14	+1·7 ± 3·9	+0·69 ± 0·18	−0·24 ± 0·13	−0·11 ± 0·06	−0·08 ± 0·07
15–18	−1·7 ± 2·4	+0·34 ± 0·03	−0·16 ± 0·17	+0·06 ± 0·11	−0·08 ± 0·03
LOW-ENERGY WINTER RATION					
'Low-yield' animals					
Winter feeding	24·9 ± 2·5	4·16 ± 0·29	8·60 ± 0·15	4·42 ± 0·08	3·22 ± 0·18
Grazing, days 7–10	+5·9 ± 1·2	+0·05 ± 0·10	+0·11 ± 0·07	+0·08 ± 0·08	+0·12 ± 0·07
11–14	+7·4 ± 1·2	−0·05 ± 0·15	+0·20 ± 0·11	+0·07 ± 0·06	+0·17 ± 0·07
15–18	+7·9 ± 1·9	−0·33 ± 0·11	+0·36 ± 0·14	+0·12 ± 0·12	+0·32 ± 0·07
'High-yield' animals					
Winter feeding	34·9 ± 4·6	3·62 ± 0·12	8·44 ± 0·37	4·50 ± 0·24	3·00 ± 0·16
Grazing, days 7–10	+5·7 ± 1·6	+0·50 ± 0·36	+0·00 ± 0·12	+0·01 ± 0·14	+0·13 ± 0·07
11–14	+7·3 ± 1·4	+0·70 ± 0·76	+0·11 ± 0·02	−0·07 ± 0·08	+0·22 ± 0·07
15–18	+7·5 ± 2·2	−0·05 ± 0·15	+0·30 ± 0·01	+0·04 ± 0·10	+0·30 ± 0·11

Solids-not-fat

A noteworthy feature of the results is a highly significant ($P < 0·01$) effect of the plane of energy nutrition during the winter feeding on the group responses in S.N.F. over the three grazing test-periods. The mean S.N.F. percentage of the 'low-energy' groups rose, eventually, by 0·33 and that of the 'medium-energy' groups by 0·11, whilst that of the 'high-energy' groups fell by 0·12. The differences between the 'high-energy' and the 'low-energy' groups were significant ($P < 0·01$) in each of

the test-periods, and the differences between the 'high-energy' and 'medium-energy' groups were significant ($P < 0{\cdot}01$) in the last two.

The level of milk yield influenced the group mean responses in S.N.F. percentage consistently, the 'low-yield' animals showing a larger increase, or smaller decrease than the 'high-yield'. This effect reached significance ($P < 0{\cdot}05$) only in the second grazing test-period.

Lactose

It is generally accepted (Burt, 1957) that level of feeding, with the exception of marked underfeeding, has little effect on the lactose content of milk, and the results presented here are in agreement. The changes in lactose percentage with grazing were small: the mean increase was greater for the 'low-energy' than for the 'high-energy' groups in each test-period, but did not reach significance. The 'low-yield' groups, as a whole, showed a larger increase in lactose than the 'high-yield' cows—an effect which was significant in the first two periods and contributed to the increase in S.N.F. The animals in the 'low-yield' groups were further advanced in lactation than those in the 'high-yield' groups and it is likely that the increases in milk yield associated with an improvement in the energy nutrition may have led to a reversal of the usual fall in lactose content with advancing lactation.

Table 2. *Mean values, with standard deviations, for each group of four cows, for nitrogen distribution in the milk for the last* 4 *days of winter feeding, and the group mean changes, with standard deviations, for days* 15–18 *of grazing*

	Total N, mg/100 g	Casein N, mg/100 g	β-lacto-globulin N, mg/100 g	Residual albumin N, mg/100 g	Proteose + globulin N, mg/100 g	Non-protein N, mg/100 g
NORMAL ENERGY WINTER RATION						
'Low-yield' animals						
Winter feeding	534 ± 56	398 ± 50	42 ± 8	26 ± 3	33 ± 12	34 ± 1
Grazing	+19 ± 25	+10 ± 30	+6 ± 7	+8 ± 4	−2 ± 11	−4 ± 3
'High-yield' animals						
Winter feeding	503 ± 27	364 ± 25	44 ± 5	30 ± 5	30 ± 5	35 ± 2
Grazing	+16 ± 22	+14 ± 8	+4 ± 3	+5 ± 3	0 ± 3	−5 ± 1
HIGH-ENERGY WINTER RATION						
'Low-yield' animals						
Winter feeding	505 ± 19	368 ± 24	43 ± 5	33 ± 5	26 ± 2	36 ± 6
Grazing	−7 ± 13	−3 ± 16	+2 ± 10	−1 ± 9	0 ± 4	−4 ± 8
'High-yield' animals						
Winter feeding	519 ± 30	388 ± 40	40 ± 6	33 ± 5	28 ± 4	30 ± 1
Grazing	−10 ± 2	−16 ± 4	+3 ± 11	+1 ± 6	+2 ± 4	0 ± 3
LOW-ENERGY WINTER RATION						
'Low-yield' animals						
Winter feeding	507 ± 29	370 ± 32	48 ± 5	27 ± 5	28 ± 4	34 ± 4
Grazing	+48 ± 13	+36 ± 19	+12 ± 9	0 ± 10	+3 ± 5	−3 ± 2
'High-yield' animals						
Winter feeding	469 ± 28	344 ± 29	38 ± 3	26 ± 2	28 ± 4	32 ± 3
Grazing	+50 ± 21	+33 ± 16	+6 ± 4	+12 ± 12	+2 ± 12	−3 ± 3

Total nitrogen and the nitrogen fractions

The effect of the winter plane of energy nutrition on the changes in the protein (total N × 6·38) content of the milk was highly significant ($P < 0{\cdot}01$) over the test-periods. The mean protein percentage for the 'low-energy' groups increased progressively with grazing, the maximum increase of 0·31, or 10% of the pre-grazing level accounting for most of the increase in S.N.F. The corresponding increase in true protein N (total N less non-protein N) of 52 mg/100 g was equivalent to 11% of the pre-grazing level. By the last test-period the mean protein percentage for the 'normal-energy' groups increased by 0·12, and that for the 'high-energy' groups decreased by 0·07, seven out of the eight animals showing a fall. The very small differences in the responses of the 'low-yield' and 'high-yield' groups were not significant.

As Table 2 shows, whenever there was a change in total N with grazing, a large part of the change was accounted for by the casein N, and there was, in general, a concomitant change in the β-lactoglobulin N and the residual albumin (α-lact-albumin and traces of serum albumin) N, but not in the proteose plus globulin N. The changes in the small amounts of β-lactoglobulin N and residual albumin N must be interpreted with caution, but they appear to be greater in proportion to the pre-grazing levels than the changes in casein N. These results for N distribution confirm the earlier findings of Rook & Rowland (1959) on the changes with grazing.

DISCUSSION

When the cows were transferred to good, spring pasture with grazing allowed to the limit of appetite, from winter feeding at a low plane of energy nutrition, there was an appreciable increase in the S.N.F. content of the milk, when from a normal plane a moderate increase, and when from a high plane a fall, with corresponding changes in milk yield. These additional observations strongly support the view recently expressed by Rook & Rowland (1959) and Waite *et al.* (1959) that when a rise with spring grazing does occur in the milk S.N.F. and protein it probably results from an improvement in the plane of energy nutrition. Spring grass of high quality is a concentrated food, in terms of the starch equivalent of its dry matter, and there is no need to postulate a special effect of any constituent.

When the 'normal-energy' groups were on the winter feeding they received a daily average of 16 lb S.E./cow and the 'high-energy' groups 20 lb. The changes with grazing in the milk yield and S.N.F. of these groups, if due solely to a rise and a fall respectively in their S.E. intakes, would imply average S.E. intakes at pasture well within the range of 16–20 lb, which would be achieved by cows consuming daily about 28 lb of herbage dry matter of S.E. about 65–70, which are reasonable estimates.

Though any increase in the S.N.F. content of milk with grazing is essentially an increase in the protein content, because of the associated increase in milk yield the production of protein and lactose is increased, but the synthesis of protein is increased to a proportionately greater extent. In the cow the main sources of energy are the volatile fatty acids, acetic, propionic and butyric, produced by microbial fermenta-

tion of the food in the rumen. Rook & Balch (1959) have recently suggested from the results of infusion experiments with milking cows that these acids have separate effects on milk secretion, an increase in rumenal acetic acid production stimulating milk yield and the synthesis of fat, protein and lactose, whereas an increase in propionic acid production specifically stimulates milk protein synthesis and depresses fat synthesis.

Of particular interest, therefore, are the findings of Annison, Lewis & Lindsay (1959) with sheep, that on changing from winter feeding to spring grazing there was a considerable increase in the rumenal concentrations of acetic and propionic acids and an increase in the ratio of propionic acid to acetic acid. Similar changes can be expected to occur when the cow is transferred from a low or normal energy winter ration to good spring grazing, and if these can be demonstrated definite progress will have been made in understanding the nutritional origin of the increase in milk yield and S.N.F. content with grazing.

It is known (Balch *et al.* 1955; Balch & Rowland, 1957) that high-energy winter rations containing, as commonly, flaked maize, and such as were given to the 'high-energy' groups of cows in the present investigation, also increase the ratio of propionic acid to acetic acid in the rumen, and it seems likely that high-energy winter feeding and spring grazing are strictly comparable in their effects on rumen fermentation and milk yield and composition.

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Reviews of the progress of dairy science

Section E. Diseases of dairy cattle

(*Received* 10 *June* 1960)

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I. MASTITIS

A. *Control*

During the past two years important papers have been published showing that faulty milking technique is one of the major causes of mastitis, and the emphasis has been not so much on hygiene as on the repeated mild trauma of the udder that the milking machine may cause. Wilson (1958*a*) points out that, under modern conditions of milking, cows are exposed to risk of infection at least twice daily throughout lactation, and it is considered that infection will proceed to invasion, more especially with the less-adapted parasites, only when some external factor has lowered the resistance of the cow. The most common factor in the lowering of resistance is the milking technique, and the part of the milking machine most likely to be involved is the liner, particularly its bore, its tension and the softness of its mouthpiece. There is some doubt as to whether pulsation rate and vacuum level are important, but inadequate vacuum reserve, with consequent surging, has definitely been implicated in a number of cases. A technique must be adopted that ensures that every cow has the cluster removed as soon as milking is finished. Maffey (1959) reports on methods for the control of aspecific types of mastitis. An investigation was made into 234 herd outbreaks of mastitis and 198 of them were assessed as caused by organisms other than *Streptococcus agalactiae*, and were, as a rule, not of the severe acute type. Control measures, which included a change to a more efficient milking technique and adjustments to the milking machines, were put in force, and resolution of the condition was obtained in 80 % of the herds in 4–6 weeks. A bulletin entitled *Machine Milking* has been produced by the Ministry of Agriculture, Fisheries and Food (1959), in order to provide a modern authoritative and comprehensive work on the milking machine. In addition to chapters on the anatomy and physiology of the udder, and on the nature and use of the milking machine, there is a chapter on milking machines and mastitis which presents a critical review of the literature on this subject. Schipper (1959) points out that although inefficient management has been recognized for a long time as a predominant initiating cause of mastitis, it has been overshadowed by extensive investigations relating to causative agents, and the search for anti-

* The years covered by this review are mainly 1958 and 1959.

bacterial preparations that would compensate for the laxity in management of the inefficient cowman. Schipper & Olson (1959) stress the need for a simple mastitis prevention programme based on practical dairy management, without the prophylactic use of antibiotics. Noorlander & Schalm (1959) have found that at least half of all the pulsators they have tested have shown signs of wear or malformation due to the accumulation of foreign material on moving parts. Correction of faults or replacement had a beneficial effect on udder health leading to a reduction of the mastitis problem. Yoshikawa & Okada (1958) found that rough handling of the udder caused large numbers of leucocytes and epithelial cells to appear in the milk. Edwards (1958) mentions the use of too many milking machines per man as a cause of mastitis. Thörne (1958) stresses the importance of environmental factors, such as unsatisfactory hygiene, faulty milking technique and the poor care of animals, in the establishment of staphylococcal mastitis. Bratlie (1958) states that although milking at a high vacuum level (50 cm Hg) resulted in a greater flow of milk and slightly more rapid emptying of the udder than at a low vacuum level (35 cm Hg) it caused more damage to the teats and increased the incidence of staphylococcal mastitis.

Ochi & Katsube (1958), after classifying some of the organisms isolated from bovine udders, came to the conclusion that, since the causative agents of mastitis are the organisms found in the normal udder flora, mastitis is a disease caused not merely by the organisms themselves, but requires predisposing factors which allow the multiplication of these organisms.

An investigation into the efficiency of various methods of disinfecting milking units in the control of mastitis due to *Str. agalactiae* has been made by Davidson & Slavin (1958). None of the methods tried achieved complete disinfection and it is suggested that complete disinfection is not possible in the short time available in the milking shed. Circulating hot water through the unit was found to be the best method but was expensive; rinsing with running water gave results as good as or better than treating with disinfectant, but all methods were unreliable when the secretion contained sticky purulent material.

B. *Treatment*

Fewer papers referring to the treatment of mastitis were published in the period under review than in the periods covered by previous reviews of this series (Blackburn, 1956, 1958). Murnane & Munch-Petersen (1959) report that attempts to eliminate *Str. agalactiae* from three herds were not successful. All quarters of all cows were treated with penicillin, and in one of the herds intensive disinfection measures were introduced and resistant animals were culled. Infection was minimized for a long period but not completely eliminated. They give as the possible reason for failure the re-introduction of infection from the environment, such as external surfaces of the udder, hand, utensils and fittings. A comparative study of three methods of control of mastitis due to *Str. agalactiae* has been made by Vallis (1958*a*). The first method consisted of early diagnosis and treatment, good preventative and hygienic measures and elimination of animals resistant to treatment. The second method was the same except that animals resistant to treatment were left on the farm. In the third method all cows were treated when they dried off. This was a three-year

experiment including twenty-seven farms with about 1000 cows. The first method was completely successful in eliminating *Str. agalactiae*, the second reduced the incidence by only about one-third and in the third method there was a two-thirds increase. Delli Quadri, McIntyre, Porter & Haight (1959) had more success using intramammary treatment with penicillin against *Str. agalactiae* than with the parenteral use of penicillin. Bacteriological examination of the milk from 1414 cows in forty-seven herds in the Turin Province, Italy, made by Pina (1958) revealed that 613 (43·3 %) were infected with *Str. agalactiae*. A month later after treatment with penicillin only 21 % were infected. A second treatment was not on the whole satisfactory, and he considers that control is bound up with strict hygiene.

It has been found by Throop & Swanson (1958) that streptococci from udders repeatedly treated with penicillin did not become resistant, though resistance could be induced *in vitro* by growing the organism in the presence of penicillin. The organisms were not typed. Infection with a strain of *Streptococcus uberis* from an udder that was not cleared by penicillin treatment was easily controlled in another experimentally infected udder. This suggests that the udder not cleared of infection by penicillin was resistant to treatment, although the organisms were susceptible. Udders which were resistant to treatment, although the organisms were susceptible to the antibiotic used, have also been encountered by Blackburn (1959). In his work, eight quarters which for one or more lactations showed repeated infection of the milk with coagulase-positive staphylococci, in spite of treatment with streptomycin, were affected with botryomycosis. It is possible that such lesions can cause the milk to become re-infected.

Reports on treatment of mastitis with neomycin come from Durrell (1959) who used it in combination with one or more other antibiotics, and from Marookian (1959) who used it in combination with thiostrepton. Redaelli & Rosaschino (1957) have tested the *in vitro* and *in vivo* activity of various antibiotics against *Cryptococcus neoformans*. In experimental cryptococcal mastitis using actidione or nystalin it was possible to sterilize the secretion but there was no change in the clinical picture. McAuliff, Phillips & Steele (1958) have used polyvinylpyrrolidone (HPX), which is derived from high-pressure synthesis of acetylene, in conjunction with penicillin and streptomycin in the treatment of mastitis caused by *Str. agalactiae*. Bacteriological recovery was obtained in 94 % of cases compared with 67 % of cases using the antibiotics alone. Keller & Boller (1959) report that the use of prednisone in the treatment of mastitis appeared to have no beneficial effect. Vallis (1958*b*) injected penicillin into the udder tissue of quarters infected with *Str. agalactiae* which were resistant to other forms of treatment. In twelve quarters with clinical lesions nine were clear of infection a month later but none was clear 6 months later. V. Heimburg (1959) treated cows with subacute mastitis by stripping the infected quarter and introducing 10–18 ml of blood taken from the jugular vein. The quarter was stripped again in 24 h and then four or five times daily until the secretion became normal. He claims that a return to normal lactation occurred in twenty-two out of thirty-two quarters. Vigue (1959) prefers the use of aqueous solutions or emulsified pastes to ointments or oily vehicles for the infusion of antibacterial substances into the udder. He recommends the addition of small quantities of surface-active agents such as 'Tween-80' to the infusion to enhance spreading and penetration.

The level of penicillin in the milk after intramuscular injection of benzathine penicillin V has been studied by Hollister, Huebner, Boucher & DeMott (1959). Following one intramuscular injection the level in the milk rose and fell more slowly than the level in the blood. In an atrophied quarter in late lactation penicillin continued to appear in the milk, and fibrotic quarters produced milk showing as high or higher concentrations than non-fibrotic quarters, but the udder secretion of dry cows showed no penicillin. Inflammatory exacerbations in the udder interfered with the appearance of penicillin in the milk. Redaelli (1958) has studied the level of penicillin after intravenous or intramuscular injection in the milk of healthy cows and cows with streptococcal mastitis. He found the levels of penicillin to be practically identical in the milk from healthy and diseased udders.

A few papers refer to testing of mastitis organisms for resistance to antibiotics. Milunović (1958) tested 522 freshly isolated strains of *Str. agalactiae* and found four of them resistant to penicillin, 189 to streptomycin, four to chlortetracycline, three to chloramphenicol and three to oxytetracycline. Abel & Pinochet (1958) have tested the sensitivity to various antibiotics of strains of *Staphylococcus aureus* from cases of bovine mastitis and found sensitivity to be high. In fact it varied from 89 % of the strains being sensitive to streptomycin and chlortetracycline to 100 % being sensitive to erythromycin. Månsson (1958), on the other hand, testing 150 strains of staphylococci isolated from clinical cases in which antibiotic therapy was ineffectual, found only 42 % of the strains sensitive to chlortetracycline, 63 % to penicillin, 75 % to chloramphenicol and 85 % to erythromycin. The antibacterial activity *in vitro* in milk of fourteen proprietary preparations containing single or combined antibiotics and other drugs has been tested by Price, Zolli, Gallian & Shea (1958). Products containing penicillin, erythromycin or the tetracyclines were most effective against streptococci and staphylococci. Against pseudomonous organisms and *Escherichia coli* only a small number of preparations brought about demonstrable inhibition in milk, preparations containing oxytetracycline and polymyxin B being the most active, but chlortetracycline, dihydrostreptomycin, streptomycin and mercury showed some activity. Drury (1958) reports that in *in vitro* tests penicillin, streptomycin and the tetracyclines were the most consistently effective against organisms causing mastitis.

In a letter addressed to the medical profession Wilson (1958*b*) gives figures to show that there is no evidence of increasing resistance of mastitis staphylococci to antibiotics other than to penicillin. He compares tests using the sensitivity disk method made on 500 strains isolated in the years 1955–56 and 162 strains in the year 1958. The figures given for the two periods are for pencillin (2·5 units) 40·6 and 68 % respectively; for streptomycin (80 mg) 1 and 1 %; for chloramphenicol (100 mg) 0 and 1 %; for chlortetracycline (100 mg) 0·2 and 0 %; for oxytetracycline (100 mg) 0·8 and 0·4 %. He points out that herd treatment for staphylococcal infection is seldom practised, and that whilst there is not a sharp dividing line between staphylococci of bovine and human origin, there are definite differences, and it is improbable that milk represents a danger to man as a source of antibiotic-resistant strains. In the course of a study of 456 mice infected with one or other of two strains of coagulase-positive staphylococci Bliss & Alter (1959) found that streptomycin resistance occurred only rarely; six mice yielded substrains of high resistance and seven mice substrains

with low resistance. About half the mice received no treatment with streptomycin, and from them two of the very resistant strains were derived . From this observation it was concluded that high resistance is a result of spontaneous mutation.

C. *Bacteriology and diagnosis*

Streptococcal mastitis. A modification of the CAMP test has been described by Pedersen (1959) in which the β-toxin is incorporated in the blood agar medium, instead of being derived from a β-toxin producing staphylococcus inoculated in a restricted zone on the plate. Plommet (1958) has confirmed that a selective T.K.T. medium and the CAMP reaction can be successfully used for the detection of *Str. agalactiae.* Sethi, Hale & Plastridge (1958) examined 543 milk samples from nine herds infected with *Str. agalactiae* before and after incubation of the samples. Plating of incubated milk revealed the organism in 161 samples, and the organism was detected in 133 of these samples using unincubated milk. Jensen, Klastrup, Rømer, Sørensen & Terp (1958) and Rømer, (1959*a*) report that the presence of group B-, C-, G- and L-streptococci in a herd can be confirmed by examination of samples taken from the milk cans as they leave the farm.

Mastitis associated with *Str. pneumoniae* has again been reported. Smith & Stables (1958) describe a severe outbreak of acute mastitis due to *Str. pneumoniae* type III affecting six to twenty-five cows in a herd. Systemic treatment with penicillin was a satisfactory form of treatment. The organism was not recovered on primary blood agar plates incubated aerobically. Further studies indicated that an atmosphere of hydrogen and carbon dioxide was not required for its growth. All that was needed was a moist atmosphere as in an anaerobic jar. Maclachlan, Wilson & Stuart (1958) give details of a serious outbreak of mastitis due to *Str. pneumoniae* type III in about half the animals in a herd of forty-nine cows. The first cases occurred in low yielders which were milked first at the afternoon milking. There was obviously a very low standard of hygiene, which may have been the reason why so many cows became affected. The causative organism was peculiar in its growth requirement in that it would grow only in the presence of CO_2 in the media used. It was subsequently shown that it would grow aerobically when another brand of peptone was used. Treatment of well-established cases did not succeed in restoring the milk yield but every case responded well to large doses of penicillin given via the teat canal. Rømer (1959*b*) described six cases of *Str. pneumoniae* mastitis occurring in six herds.

Staphylococcal mastitis. The use of samples taken from bulk milk cans for the detection of staphylococcal mastitis in herds has been studied by Klastrup (1958). He used a meat medium infusion peptone agar with 6·25 μg/ml of Polymyxin B and 5% calf blood, a medium which had previously been shown to inhibit Gram-negative contaminants. A single can sample detected staphylococcal infection of 81·2% of 185 infected herds. To differentiate pathogenic from non-pathogenic staphylococci, Nyiredy (1958) describes a violet-red agar medium containing 1% mannitol, 5% defibrinated blood and 4% of a saturated solution of bromcresol red, by means of which haemolysis and mannitol splitting properties can be observed. He suggests that the coagulase test should also be used for the exclusion of the few haemolytic and mannitol splitting saprophytes. Sevel & Plommet (1960) have modified the tellurite-glycine agar of Zebovitz, Evans & Niven (1955) mentioned in

a previous review of this series (Blackburn, 1956) by the addition of sheep erythrocytes and the CAMP factor to show haemolysis. This medium allows staphylococci to be isolated and identified from individual or can milk samples.

A study of the staphylococci associated with the bovine udder has been made by Reid & Wilson (1959). They examined seventy cultures, fifteen from acute cases, twenty-nine from chronic cases and twenty-six from normal udders. On the whole they found no significant differences between the staphylococci from chronic cases and from normal udders, but those from acute cases were frequently more pigmented and had, as a rule, a higher capacity for peptonization of milk and lypolytic changes. In addition 73·3 % of them produced opacity in egg-yolk medium compared with 6·9 % of the staphylococci from chronic cases and 7·7 % of those from normal udders. Redaelli & Rosaschino (1958) found that only 24·1 % of the organisms isolated from 174 cases of staphylococcal mastitis could be identified as pathogenic strains of *Staph. aureus*. After a study of 678 strains of micrococci isolated from milk samples, Nakagawa (1958) concluded that there was no significant correlation between pigment production and mannitol fermentation on the one hand and the ability to produce coagulase or haemolysis on the other.

Staphylococcal mastitis in an individual herd has been studied by Thörne & Wallmark (1960). Coagulase-positive strains isolated from the udder secretions were almost consistently of a uniform phage type and identical strains were also repeatedly demonstrated on the skin of udders, on the floor of the stalls, and on the tubing and teat cups of the milking machines. Coles & Eisenstark (1959) have studied the lysogenicity in *Staph. aureus* of animal origin, and also the use of human typing strains and adapted-human typing phages in typing *Staph. aureus* of animal origin, particularly those isolated from the bovine udder.

Edwards & Smith (1959) have examined sera from the three herds at Compton for α and β antitoxin. In one herd in which infection of the udder is, as a rule, with phage type 3A which does not produce β haemolysis *in vitro*, the rise in β antitoxin levels did not increase with age. In the other two herds infected with phage types 42D and 78 respectively, which both produce α and β haemolysis *in vitro*, there was an increase with age. They also examined the milk for level of antitoxin, and during lactation only six of 105 samples showed an antitoxin level of 0·25 unit per ml or over. At calving α and β antitoxin were present in all but two of twenty-five cows, and were present for only 3–6 days, but in those developing mastitis these toxins were there for a longer period.

Organisms other than streptococci and staphylococci. A histological examination of the udders of thirty cows with mastitis associated with *E. coli* has been made by Fey & Stünzi (1958) and twenty-two of them were found to show necrosis of the udder tissue. Organisms isolated from the udder tissue or mammary secretion were injected intracutaneously into rabbits to find whether they produced necrotoxin. There appeared to be no relationship between the presence of necrosis in the udder tissue and the formation of necrotoxin by the organisms. Cole & Easterbrooks (1958) observed leucopenia in all of twelve cows with experimental acute Klebsiella mastitis. Goyen & Cursan (1958) report an outbreak of mastitis in a twelve-cow herd in Normandy due to *Aerobacter aerogenes*. Treatment with streptomycin was successful.

An outbreak of mastitis caused by *Serratia marcescens* is described by Barnum,

Thackery & Fish (1958). Seventeen quarters of eleven cows were affected. Inoculation of a culture containing 25000 organisms into a quarter caused mastitis resembling that which occurred naturally. It was found that of the antibiotics tried only neomycin eliminated the organism. They classify the infection as a mild chronic mastitis with no systemic reaction but with a drop in milk yield. The reaction of the quarter was cyclic in that clots were found in the milk at intervals. Blum, H. (1958) reports two cases of mastitis in different herds from which *Pasteurella multocida* was isolated. Obiger (1959), discussing *Corynebacterium pyogenes* mastitis, reviews the problems of insect-borne disease. Hengl, Kasza & Kemenes (1958) report that infection of dairy cows with *Leptospira pomona* or *L. hyos* may be manifested by a sudden outbreak of mastitis. All four quarters of an udder may be affected at the same time, but the condition usually clears up in a few days without treatment. Heidrich & Grossklaus (1959) report two cases of acute mastitis from which *Clostridium welchii* was isolated. An outbreak of mastitis affecting thirty-six quarters of forty-three cows, and attributed to *Sphaerophorus* species, is described by Simon & McCoy (1958). Histological examination of the udders showed acute lobular mastitis. In the udder the organism was encapsulated but no capsule was seen on culture. Although the organisms were sensitive *in vitro* to various antibiotics, treatment was not satisfactory. Mastitis due to *Nocardia* (*Actinomyces*) *asteroides* has been referred to in previous reviews of this series (Blackburn, 1956, 1958). Pier, Gray & Fossatti (1958) give a fuller report of an outbreak in which the organisms were cultured from the milk of twenty-eight cows (18 %) in a dairy herd. Naturally infected cows showed extensive fibrosis of the udder and palpable granulomatous nodules. Dafaalla & Gharib (1958) have reported a case in the goat, and Ditchfield, Butas & Julian (1959) have described a case of chronic granulomatous mastitis in a cow caused by *N. brazilianis*.

It has been found by Redaelli (1957*a*) that strains of *Cryptococcus neoformans* which proved virulent for laboratory animals were capable of producing in goats and cows experimental mastitis that was similar to the natural disease. He is therefore of the opinion that the conception that infection with this organism could only be in direct relation to antibiotic treatment must be modified. On the other hand, the same author (1957*b*) found that various genera of microfungi, such as *Candida*, *Torulopsis* and *Saccharomyces* isolated from animals with mastitis, failed to cause mastitis when tried experimentally. *C. tropicalis*, when injected into the udders of cows or goats along with a large dose of penicillin, caused an inflammatory reaction which was much more marked than the reaction obtained by injecting the organisms alone. Loken, Thomson, Hoyt & Ball (1959) describe an outbreak of mastitis in a herd caused by *C. tropicalis* which occurred after fifteen cows had been treated for chronic mastitis associated with *Str. agalactiae*. Quarter samples taken from the fifteen cows showed that forty-five quarters were infected (75 %). The infection was said to be self limiting, and after 2 weeks only one quarter showed clinical signs. Barbesier (1958) reports that a fungus having the morphological, cultural and biochemical properties of *C. krusei* was isolated from the secretion of two quarters affected with acute mastitis which failed to respond to antibiotic therapy.

The viruses of vesicular stomatitis, vaccinia and Newcastle disease were infused into the udders of cows which were free from streptococci or staphylococci by Easterday, Hanson & Simon (1959). They point out that if the cows used in this study had

been encountered in the field, the reaction caused by the viruses, as shown by the increased cell count and the Whiteside test and by the absence of organisms, would have led them to describe the cases as being examples of ideopathic mastitis. They say, however, that this study neither substantiates nor questions the role of viruses in mastitis. Bannister, Boulanger & Rice (1959) report that, after an emulsion containing virus of the psittacosis-lymphogranuloma group had been infused into the udder of a cow, severe mastitis was produced. Evidence was obtained of multiplication of the virus and its excretion in the milk.

Diagnosis. There are numerous papers referring to various aspects of the cell count of milk. Guallini & Vallis (1957) made a statistical analysis of total cell counts of milk from 288 'healthy' quarters and 216 mastitis quarters. The range of cell counts obtained from the milk from 'healthy' quarters casts some doubt as to whether the quarters were healthy. The conclusions drawn from this work, however, appear very sound, viz. that quarters giving milk with less than 80000 cells per ml are normal, and that quarters giving milk with over 1000000 cells are definitely mastitic, and that where the intermediate ranges are observed mastitis may or may not be present.

Easterday, Simon & Hanson (1958) have compared the modified Whiteside test with the cell count and bacteriological findings for quarter samples of milk from five herds. They found that there was a general correlation between the degree of Whiteside reaction and the number of leucocytes in the milk samples, but that specific degree of reaction could not be correlated to a specific number of cells. They found about three times as many samples infected with coliform organisms as with any other organism, and only 8% of these samples were positive to the Whiteside test. This leads one to suspect, especially as they incubated the samples before plating, that the majority of these organisms were contaminants. Strozzi & Negretti (1959) have also compared results obtained by the Whiteside test and the cell count. They found a good parallel between the methods but this did not allow the substitution of the easier Whiteside test for the cell count. In the previous review of this series (Blackburn, 1958) it was mentioned that in Britain a detergent selling under the commercial name of 'Teepol' had been found satsifactory for use in the California Mastitis Test (C.M.T.). McKenzie & Cameron-Mackintosh (1958) report that this substance gave a reasonably clear cut reaction with milk samples containing less than 100000 cells/ml or more than 500000 cells/ml. Negretti (1959) gives details of a modification of the Whiteside test using antiformin instead of sodium hydroxide. A device for measuring the electrical conductivity of milk was compared with sediment and thibromal tests and with the Whiteside test by Baumgartner & Walser (1959). They found it as good as the other tests for quarter samples, but unsuitable for samples of mixed milk. Schönherr (1958) describes a commercially produced paper-strip, impregnated in four places with a yellow indicator dye. A few drops of milk are put on each spot and the changeof colour noted. A bluish green or green colour darker than a line printed on the paper is indicative of some secretory disorder.

Paper electrophoretic studies of whey protein fractions of milk from cows with mastitis have been reported. Weigt (1959) has shown the shift of these fractions in various stages of chronic and acute mastitis associated with streptococci. A fraction migrating at the same rate as blood serum albumin was found by Lecce & Legates (1959) in milk from quarters with mastitis. Relative increases in the amount of

immune globulins were also found. Nani & Redaelli (1958*a*) have shown that the lactalbumin and lactoglobulin fraction increased. A fraction similar to blood serum albumin was present in colostrum, in late lactation milk and in milk from quarters severely affected with mastitis. Nilsson (1958) was of the opinion that the ninhydrin-positive fraction of whey protein, appearing in milk from cows with mastitis, was probably immune globulin locally produced in the infected udder.

D. *General*

Yield and composition. The effect of udder infections on milk yield and milk solids has been studied by O'Donovan, Dodd & Neave (1960). Using data from fifty cows affected mainly with *Staph. pyogenes* they found that lactation yields of milk, solids-not-fat and fat were depressed by 10, 11 and 12% respectively in the presence of infection. They suggested that the depression would have been greater in the absence of antibiotic therapy for mastitis. Rowland, Neave, Dodd & Oliver (1959) report on the effect of *Staph. pyogenes* infections on milk secretion. From samples drawn from ninety-two infected quarters compared with non-infected quarters it was found that during the period of infection the milk yield was decreased by 15·3 ± 2·5%, the butterfat percentage by 0·19 ± 0·03, and the solids-not-fat percentage by 0·20 ± 0·02. It appeared that the overall effect of infection on milk composition was very small. Arima, Saito, Nakagawa & Yusa (1958) have confirmed that there is a decrease in the lactose content of the milk and an increase in the chlorides and non-casein nitrogen in udders affected with subclinical mastitis. Renk & Teute (1958) have found that mastitis milk had a lower content of total protein, casein and albumin than normal milk but a higher globulin content. Redaelli & Crespi (1958) found that milk from quarters with mastitis associated with *Str. agalactiae* had a lower acidity and higher electrical conductivity than milk from healthy quarters of the same cows. No significant differences were found between the specific gravities, viscosities or surface tensions of the milks. Nani & Defranceschi (1957) found that in *Str. agalactiae* mastitis all the vitamins of the B complex appeared to increase during the clinically active phase of the condition. Nani & Redaelli (1958*b*) believe that the increase of B6 and B12 is probably due to a liberation of the free vitamin at the expense of the combined form. The phosphorus content of eleven samples of milk from healthy and mastitic quarters of the same animal was determined by Caimi & Redaelli (1958), who found that the total and esterified phosphorus fractions were significantly lower in the milk from the infected quarters.

Incidence. A few papers refer to the incidence of mastitis. In Milan province Vallis & Rosaschino (1958) examined thirty herds and found that infections with *Str. agalactiae* (20·8%) exceeded those caused by other organisms (6·2%). The incidence varied according to whether methods of controlling *Str. agalactiae* infections were applied. Where eradication was being attempted, infection with *Str. agalactiae* was 1·1% and with other organisms 10·5%; where animals were examined every three months and infected animals treated the figures were 10·3% and 8·5% respectively; where treatment was applied only to dry cows the corresponding values were 39·2 and 3·1% and where no control was attempted they were 36·3 and 1·9%. Kjos-Hanssen (1959) reports that during the period 1949–59, 7009 cases of clinical mastitis in the Rozaland county of Norway were examined. The incidence of *Staph. aureus*

mastitis increased from 20·5 % of the clinical cases in 1949 to 48 % of those in 1959, while *Str. agalactiae* mastitis fell from 34 to 10·5 %. Bacteriological and cytological examination of milk from 344 cows in twenty herds in the southern province of Mantua has been made by Signorini (1957), who observed that 44 % of the animals were infected with *Str. agalactiae*, and that 95 % of the herds contained infected animals. Deb (1959) found the incidence of subclinical mastitis in an Indian dairy herd of 476 Hariana cows and seventy-six buffaloes to be just over 30 %. The incidence in cows and buffaloes was similar (30·6 and 30·5 %) respectively.

Experimental mastitis. The experimental production of staphylococcal mastitis in the goat is reported by Derbyshire (1958*a*, *b*). Strain 201 isolated originally from a naturally occurring case of bovine mastitis was used. Living inocula produced mastitis in all but one goat in which the dose was very small. The reactions varied from gangrenous mastitis to severe non-gangrenous forms and even to mild mastitis in one goat. Examination of the secretion from the gangrenous cases showed large numbers of neutrophyls for 24–48 h but the numbers decreased as the gangrenous changes developed. Histopathological examination showed that 6 h after inoculation massive numbers of staphylococci had penetrated the alveoli and were widely distributed in the mammary tissue, and that there was widespread migration of neutrophils from dilated capillary blood vessels into the alveoli. From 24 h after inoculation, at the stage when the disease was recognizable clinically as 'gangrenous' mastitis, evidence of thrombus formation could be found in the interlobular veins and in the main veins at the dorsal aspect of the inoculated half of the udder. Cocci were not found in direct association with the thrombosed veins. Once thrombo-phlebitis had developed, inflammatory changes in the mammary tissue were arrested, but degeneration continued so that the alveolar structure of the lobules was lost. In a late stage of the disease widespread areas were completely necrotic. This work makes an extremely valuable contribution to the understanding of this important type of mastitis. The effect of certain mild stresses on the bovine teat canal on infection with *Str. agalactiae* has been studied by Murphy (1959). Neither repeated passing of a sterile stilboestrol saturated swab into the canal nor milking at elevated vacuum caused quarters to be susceptible to repeated exposure to the organism. Reaming of the canal, however, using vinyl tube 0·08 in. in diameter, rendered uniformly susceptible all teats which were reamed. After infection was removed by treatment with penicillin and sufficient time allowed for the keratin to be reformed, all quarters again became resistant to infection with *Str. agalactiae*. Hyde & Murphy (1959) attempted to increase the resistance of the teat canal by the use of a hyperkeratotic chemical, chlorinated naphthalene.

Using fifty-two cows with severe acute-non-gangrenous mastitis and eight cows with gangrenous mastitis, Theilen, Schalm, Straub & Hughes (1959) have studied the leucocyte response to acute mastitis. Within a few hours of onset the total leucocyte count often falls below the minimal normal level of 4000 cu.mm of blood. The extent of the 'shift to the left' was related to the severity of the mastitis, and was more extreme in gangrenous mastitis. With the appearance of the 'shift to the left' the total count increased.

Two papers refer to immunization of cows against staphylococcal mastitis. Pearson (1959) used a vaccine-toxoid preparation made from two to six freshly isolated strains

of staphylococci. In the first 21 months of the trial only one of twelve vaccinated animals contracted clinical staphylococcal mastitis compared with nine of the twelve controls. Slanetz, Bartley & Allen (1959) vaccinated cows with toxoid or with bacteria-toxoid, and found that with either of them the resistance to staphylococcal mastitis could be increased.

Two papers refer to feeding as a possible cause of mastitis. Pounden, Pratt, Frank & Smith (1958) fed fresh cut forage to one group of cattle and silage made from similar material to a second group. There was no significant difference in the incidence of mastitis in these two groups, but curiously enough there was a higher incidence in a third group which received fresh cut forage and silage alternately. Frank, Pounden, Weaver & Gilmore (1959) tested the activity of various organisms in the milk from groups of cows fed either forage crop or corn silage. With a mastitis-producing staphylococcus and a coliform organism they noted the time required to reduce methylene blue put in the milk—the longer the time required the more resistant the milk to the growth of the organism. With *Str. agalactiae* they recorded the acid production in the milk. They found very little difference in the activity of the milk in either type of feeding, but when the cows were turned out to pasture, there was a tendency in some years for the milk samples to lose temporarily their resistance to staphylococci.

Histopathological studies of thirty udders obtained from the slaughterhouse have been made by Yamagiwa, Ono, Uemura & Ida (1957). They give a very sound classification of bovine mastitis, dividing it into 'Mastitis circumspecta' and Mastitis diffusa'. By 'mastitis circumspecta' they mean that lesions of acute lobular mastitis are found focally distributed in the gland, but the term 'mastitis diffusa' signifies something more than that the lesions are more diffuse—there are, in addition, lesions in the ducts and in life the mastitis would have been quite obviously clinical. The same authors (1958) have studied the tissue reaction to corpora amylacea in the mammary gland. Minamimoto, Shibata & Ishi (1959) examined the bacterial flora from the teat and gland cisterns and from the mammary glands of 150 cows. They showed the relationship between bacteria found and the macroscopic and microscopic changes in the gland, and the distribution of staphyloccoci and streptococci in different parts of the udder. Mastitis appeared to be associated with bacteria which produced hyaluronidase.

Under the headings, predisposing factors, infection, diagnosis and control, Plastridge (1958) has reviewed some of the literature on bovine mastitis, going back for 30 years, and Nai (1957) has reviewed some of the literature published during the last 70 years on the aetiology and pathogenicity of infectious mastitis in cattle.

E. *Conclusions*

Reading through the previous reviews of this series published during the past 10 years (Blackburn, 1952, 1954, 1956, 1958) it is possible to pick out the major trends in research on bovine mastitis during this period. The elimination of infection with *Str. agalactiae* from herds has been found to be possible. It is probable that this is not so easy to accomplish in areas where the incidence is high, owing to the increased likelihood of re-introduction of infection, Although mastitis caused by this organism has been reduced in British herds by the use of antibiotics the disease is still prevalent (Wilson, C. D., 1957). In addition, mastitis caused by staphylococci and by other

streptococci has assumed a greater role, and these types of mastitis do not respond so well to antibiotic therapy. Much of this mastitis is subclinical and therefore largely ignored. In the present review evidence is given that these other types of mastitis can to some extent be controlled if the repeated mild injury caused by the milking machine is avoided. It has been pointed out that there are twenty or more kinds of infection causing mastitis (Murphy, 1956), and although any of these can be important in individual herds, 99 % of all mastitis is accounted for by infection with *Str. agalactiae*, other streptococci, staphylococci and bacilli.

There has been a great increase in the knowledge of the pathology of mastitis, and this may in part be responsible for the fact that the majority of authors are now careful to define what they mean by the term mastitis; whether they are referring to severe acute mastitis with systemic symptoms; to heat pain and swelling of a quarter; to the appearance of clots in the fore milk; to mastitis recognizable only by indirect tests or merely to infection of a quarter.

II. BRUCELLOSIS

A. *Diagnosis*

A few papers published in the period under review refer to the milk ring test (M.R.T.). Janney, Berman & Erdmann (1958) did blood tests 3–4 weeks after ring tests to evaluate the accuracy of the M.R.T. In fourteen herds the M.R.T. located seven herds which contained reactors. In four of the other seven the reactors were not producing at the time of the ring test, and the remaining three herds, each of which had been shown to have only one reactor to the blood test, were missed by the M.R.T. Roepke, Stiles & Driver (1958) stated that dairy herds which were consistently negative to the M.R.T. under a programme in which the ring tests were made half-yearly might with safety be considered as negative for the purposes of recertification of a county as 'modified-certified brucellosis free'. Bartels (1959) obtained positive reactors to the M.R.T. with the churn milk from 155 previously negative herds, and doubtful reactors in 147 previously negative herds. Individual blood and milk tests on each animal in these herds revealed at least one animal positive for brucellosis in 92 % of the herds which had given positive reactions and in 52 % of the herds which had given doubtful reactions. In work by Van Der Schaaf & Jaartsveld (1958*a*) the results of tests on the milk from artificially infected cows showed that the most reliable results with the M.R.T. were obtained when samples from one whole milking were kept at 4 °C for 1–2 days before being tested. Less reliable results were obtained when fresh milk samples were tested or when fore-milk samples were used.

Comparing the slide-aggutination test with the tube-agglutination test Khan (1958) found that the efficiency of the slide test approached that of the tube test when the titres of the samples were above 1:40, but the discrepancy was marked when the titre was less. He found also that in Khartoum the atmospheric temperature (30 °C) tended to cause a decrease in the sensitivity of the tube test, whereas incubation at 45 °C tended to increase its sensitivity compared with incubation at 37 and 30 °C. Pope & Ruedy (1959) report that in 1955, Wisconsin experienced a heat wave with temperatures of 32 °C and above, and it was observed at this time when about 10000 samples per day were being tested that there was a marked increase in the number of

suspicious titres occurring in the State-Federal Co-operative Brucellosis Eradication programme. Tests were made at 26 and 36 °C, and the higher ambient temperature significantly increased the number of suspicious and positive titres in the rapid plate-serum agglutination test. These findings indicate the need for using a uniform temperature throughout the year in all laboratories in which this test is done. From eighty-two strains Anczykowski (1957) selected S. 19 as the most suitable for the production of a standardized suspension of *Brucella abortus* for use in Poland. The same author (1958*a*) found that though a 96-h culture gave maximum growth, a 72-h culture gave a more uniformly stained suspension. Comparing the results of using stained and unstained antigen, Anczykowski (1958*b*) considered that antigen, stained with 2,3,5-triphenyltetrazolium chloride enables a more accurate differentiation between specific and non-specific reactions to be made and the reading of the final titre was easier. Bürki (1958) has found that by the agglutination test alone it is impossible to distinguish between natural infections and vaccination titres. By the combined use of the tube agglutination, complement-fixation, precipitation and Coombs tests, differentiation was possible in most instances. The joint FAO–WHO expert committee on brucellosis, in its third report (World Health Organization, 1958) states that in view of the finding that, in animals of 30 months or older which have been vaccinated as calves with S. 19, a blood serum titre of 200 International *Brucella* antibody units (I.U.) per ml indicates the same likelihood of *Brucella* infection as a titre of 100 I.U. per ml in non-vaccinated animals, the minimum diagnostic titre in known vaccinated animals should be 200 I.U. Each ampoule of the International standard contains dried material equal to 1000 I.U. and so can be reconstituted with 1 ml of distilled water to give 1000 I.U. per ml.

Stableforth (1958), discussing the methods in use for the diagnosis of bovine brucellosis, has stated that the ring test and the tube- and plate-agglutination tests, supplemented by bacteriological examination of the foetus or placenta, are the most widely used for detection of infected herds. He considers that a combination of the ring test, the whey test and cultural examination is an economical method for the control of spread and for eventual eradication of infection. The vaginal mucus test has also been used in one country where a positive result is regarded as diagnostic. Diagnosis of brucellosis in animals is also discussed by Ottosen (1958). He is of the opinion that, in cases of abortion, infection with brucella may be diagnosed by microscopic examination of cotyledons and by serum agglutination. With a combination of these two procedures an accuracy of 95 % is obtainable and therefore cultural examination and animal inoculation are unnecessary. In the control of brucellosis, however, Lembke & Frahm (1959) do not consider that the general method of the serum-agglutination test is absolutely reliable in the detection of brucellosis because infected animals may not always be discovered and thus the disease may be spread in a way that cannot readily be controlled. Using 'W' medium, Bürki (1959) isolated twenty-two strains of *Br. abortus* and one of *Br. melitensis* from placentae from animals which were negative to serological tests. Some of the cows had been vaccinated but none of the strains resembled S. 19. In Norway where *Br. abortus* infection was considered to be eradicated by 1951 and where no vaccination has been done since then, Sandvik (1958) is of the opinion that, in the serum-agglutination test, sera with titres of 1:10 but less than 50 % agglutination at 1:20 should be regarded as negative. Sera with

50 % agglutination at 1:40 should be regarded as positive. Tube-agglutination and various other tests were used by Aleandri & Gargani (1957, 1958) in two surveys of the incidence of brucellosis in bulls. They found that the frequency of agglutination titres of 1:10 to 1:40 was high, 87 % in one survey and 62 % in the second. They maintained that diagnosis of brucellosis in bulls should not be based solely on agglutination up to 1:40 unless supported by clinical evidence and other serological and biological tests.

A whole-blood agglutination test has been described by Boulanger & Smith (1958). A wire loop is used to collect blood from the ear or tail and a drop of blood is mixed with a drop of antigen on a warm glass plate. Agglutination occurs in 10–15 min with positive sera. When compared with tube-agglutination tests on over 900 vaccinated and unvaccinated cattle there were no false negative results. It is therefore recommended as a screening test for range cattle, since those which were negative to the test could be released at once and the reactors retained and bled for the tube-agglutination test. Heparin is incorporated in the antigen.

In a survey to determine the incidence of brucellosis in 147 dairy herds in Northern Ireland, Kerr, Pearson & Rankin (1958) demonstrated the limitations of the serum-agglutination test caused by the use of live vaccine. In contrast, in an experiment on eight heifers they showed that the vaginal mucus titres had disappeared 4 months after vaccination with S. 19.

Münker (1958) has compared the slow agglutination test with the complement-fixation test both in the cold and at 37 °C and also with a quantitative micro-method of the complement-fixation test. The complement-fixation test done in the cold was found to be superior to the complement-fixation test done at 37 °C, both in respect of the number of positive results and the titre obtained. The results obtained for the micro-method were comparable to those obtained by the warm complement-fixation test. The complement-fixation test has been used by Van Der Schaaf, Jaartsveld & Hesse (1959) for the identification of vaccination titres in a brucellosis eradication campaign. Twelve calves from the brucellosis-free island of Terschelling were vaccinated with S. 19 at 8–12 months old. Complement-fixation tests done on the sera of these animals were all negative 6 months after vaccination. Ten of these animals along with six unvaccinated controls were challenged with a mixture of three virulent strains of *Br. abortus* and all the controls and six of the ten heifers aborted. Complement-fixation tests on the sera of these animals remained positive for a long time, whereas the agglutination titres diminished more quickly.

Kohl & Fritzsche (1959) studied the value of the Coombs test for incomplete antibodies as an additional aid to diagnosis, comparing the titres they obtained with those of the agglutination test. On several farms, in two of which the animals had been vaccinated, tests on 3246 sera showed that animals with a Coombs titre of 1:20 or more with a tendency to rise could be considered infected, but they also found high titres on the two farms where the animals had been vaccinated. They concluded that this test reveals more reactors in an infected herd than does the agglutination test and that the test is useful provided the animals have not been vaccinated. A one-tube screen test for the detection of incomplete antibodies which is a modified Coombs test is described by Zoutendyk (1958). The entire test can be completed in a day. Hajdu (1958) gives a slide modification of the Coombs test in which samples are

examined at one dilution only. In a field trial it proved to be more sensitive than the tube-agglutination, complement-fixation, ring and skin tests.

The indirect haemagglutination test for the detection of brucellosis is described by Becht (1958). By extraction with acetic acid at 100 °C followed by precipitation by alcohol a substance can be demonstrated in *Br. abortus* which can sensitize the red blood cells of cattle. The red blood cells modified in this way are specifically agglutinated by sera from cattle with brucellosis. Papers by Parnas & Mierzejewska (1958) and Skurski (1958) refer to the opsonocytophagic test in human brucellosis.

On the subject of allergic tests for the diagnosis of brucellosis, Benedek, Hoffman & Nagy (1959) have tested the value of a brucella antigen produced by acid hydrolysis on 1474 cows injected intradermally with 0·2 ml doses. A positive reaction with an average of 97 % increase in the thickness of the skin was obtained in 387 cows. The average increase was only 13 % in the remainder of the animals. The authors suggested the simultaneous use of serological and allergy tests for routine diagnosis of brucellosis.

Agar diffusion studies of the antigens of brucella have been made by Carrère, Roux & Serre (1958) Olitzki & Sulitzeanu (1958*a*, *b*), Sulitzeanu (1958) and Bruce & Jones (1958). The technique for the preparation of *Br. abortus* and *Br. melitensis* mono-specific sera has been described by Renoux (1958*a*). Jones (1958) has given an account of the method used at the Central Veterinary Laboratory, Weybridge, for the preparation of mono-specific brucella sera. The importance of using entirely smooth cultures is emphasized. The production of mono-specific sera has been described also by Gargani & Guerra (1957). Olitzki (1959) has reported that bacterial agglutinins were removed from brucella antisera by intact bacteria or by cell walls produced by disintegration of brucella, and mono-specific antisera could be produced by adsorption with heterologous antigens. Cell-wall agglutination paralleled almost completely the agglutination of intact bacteria when cell-wall suspensions of high density were used.

B. *Bacteriology*

A number of papers have been published during the period under review dealing with selective media for the culture of brucella. Jones & Morgan (1958) have stated that serum-dextrose agar with antibiotics added gives promise of being more effective in isolating fastidious types of brucella from contaminated materials than media previously employed. The substitution of serum-dextrose nutrient agar for albimi agar in antibiotic media permits better growth of *Br. abortus* type II and comparable growth for other types of brucella. Comparison of various media for the growth and isolation of brucella has been made by Morgan (1960.) He found that a basal medium containing 0·5 % (v/v) Tween-40 was able to support growth of 80 % of the *Br. abortus* Wilson type II cultures studied. The other strains of *Br. abortus* and strains of *Br. suis* and *Br. melitensis* and intermediate types grew on the basal medium either with or without Tween-40. Neither Tween-agar medium containing the antibiotics bacitracin, polymyxin and actidione, with or without methyl violet nor albimi-antibiotic agar would support the growth of *Br. abortus* type II strains. Terpstra (1958) has recommended a modification of the medium described by Morris, which was referred to in the previous review of this series (Blackburn, 1958). This modification consists of a reduction in the concentration of 5-nitrofurfur-methyl ether. In order to find a medium for the culture of brucella species which would be cheap and easy to

prepare, Renoux (1958*b*) tested four solid media; albimi agar, tryptose agar, liver agar and potato agar. To each medium was added 10 % filtered sterile horse serum free from brucella antibodies. He concluded that potato agar with serum added was the best; it was easy to prepare, relatively cheap and kept well as a stock medium. The addition of a detergent in a concentration of 1·25 % to blood agar medium was found by Van Der Schaaf & Jaartsveld (1958*b*) to inhibit the spread of proteus species which otherwise concealed the growth of *Br. abortus*.

Manthei, Amerault & Goode (1958) have compared the efficiency of trypticase-soy and veal infusion broths for isolating *Br. abortus* from the blood of cattle. Of 353 isolations made from 2322 samples of blood, 172 were in the trypticase medium and 181 in the veal broth, 47 % of the 353 isolations being made simultaneously in both media. Babich, Plotnikova & Levina (1957) have studied dried culture media suitable for despatch to field stations, which after reconstitution with distilled water could be used for the cultural diagnosis of brucellosis.

Apparatus for growing *Br. abortus* S. 19 have been described. Sterne (1958) has given details of an apparatus, which occupies only 4·5 sq.ft in incubator area, for growing S. 19 in a dialysed medium in an aerated Cellophane tube. It is capable of producing about half a million doses of vaccine per annum. Van Drimmelen (1958*a*) describes the production of S. 19 vaccine by means of the Brucella Vortex Aerated culture apparatus (Br. V.A.C.). The apparatus delivered $1{\cdot}44 \times 10^{16}$ viable organisms in one batch, equivalent to more than 120000 doses of liquid vaccine, and took 1 week to do this. Using the Roux flask potato-agar culture method at least 4000 flasks would have been required for this amount. Propagation of *Br. abortus* S. 19 has been attempted by Hoffman, Szakmáry & Szabo (1959) using a slightly modified fermenter apparatus, as used in the production of antibiotics. A fluid medium composed of meat and liver hydrolysate, potato extract and liver dialysate containing 1 % glucose was used. After incubation for 23–25 h at 37 °C a suspension containing 16×10^{10} organisms per ml was obtained.

Huddleson (1957) has shown that the growth of brucella organisms in a mixture of peptone type of culture medium and blood is not due to growth-promoting constituents in the medium but to an agent or agents that inactivate the growth-inhibiting factor in normal blood. Other methods of inactivating this factor are acidification of the medium to a final pH of 6·0–6·2 after addition of the blood, or the addition of a cation exchange resin to the blood alone.

It has been maintained by McCullough & Beal (1958) that members of the genus *Brucella* are not labile nor subject to spontaneous species alteration. Renoux (1958*c*) and van Drimmelen (1958*b*), however, do not agree with this view. Renoux points out that the commonly accepted identification tests comprise sixty-four different identification patterns, and only three of these are accepted as being species because they were studied first. A great number of strains cannot be classified under the three species and are called aberrant strains. He suggests that a strain of brucella should be identified by the biochemical tests of Huddleson and mono-specific sera; there should be the genus *Brucella* (Meyer & Shaw) and only one species, *Br. melitensis* and that any strain with different characters should be regarded as a variety. Van Drimmelen claims that passage through a rabbit and then a guinea-pig transformed *Br. melitensis* to a strain which, in its biochemical and serological properties, was identical

with *Br. abortus*. He is therefore of the opinion that there is only one species of brucella, viz. *Br. melitensis* and that all other strains are varieties of this species. Meyer & Cameron (1959) have studied the comparative metabolism of species and types of organisms within the genus *Brucella*. They conclude that the effect of thionin and basic fuchsin upon the growth of the organisms within this genus has been the most valuable criterion for species identification, and the fact that the differentiation into three species is biologically sound is reflected in the differing metabolic patterns of the organisms within the species and types, especially when correlated with the bacteriostatic action of the dyes and of H_2S production. Meyer & Cameron (1958) have found that differences in metabolism are particularly valuable in distinguishing *Br. suis* from the other two species. The differences between *Br. abortus* and *Br. melitensis* are not as striking but, nevertheless, are characteristic. Shibata, Isayama & Murata (1958) found that strains of *Br. abortus* isolated from Jersey cows imported into Japan presented the typical characteristics of *Br. abortus*, whereas those isolated from locally bred Holsteins were more sensitive to dyes and to penicillin. Of 1017 cattle examined in Berlin abattoirs forty-one were found by Lerche & Entel (1958) to be bacteriologically positive for brucella. Thirty-nine were found to be infected with *Br. abortus* and two with *Br. suis*.

Other papers have been published referring to biochemical studies of brucellae. *Br. neotomae*, isolated from the wood rat by Stoener & Lackman (1957), has been the subject of metabolic studies by Cameron & Meyer (1958). They found by means of manometric techniques that the oxidative metabolic pattern of this proposed new species is not identical with the patterns of any of the three recognized species. A rapid method for determining carbohydrate utilization by brucella organisms is given by Pital, Cooper & Leise (1958), using paper disks impregnated with the different carbohydrates to be tested and resazurin as an indicator. Tepper & Wilson (1958*a*) have studied the fixation of $^{14}CO_2$ in *Br. abortus*. They could find no differences in the distribution of ^{14}C fixed into the amino-acids of a virulent CO_2-dependent strain and an avirulent CO_2-independent strain. The same authors (1958*b*) compared the amino-acid composition and nucleic acid content of strains of *Br. abortus* and found that neither the virulence for guinea-pigs nor the carbon dioxide requirement of a strain can be directly related to differences in the amino-acid composition or the nucleic acid content of the organisms. Whey from healthy cows and from brucellosis-infected cows was examined by paper electrophoresis by Kutáček & Kratochvíl (1958). They found that the proportion of immune globulins was greater in infected cows, and that the agglutins were chiefly associated with the faster moving immune globulin fraction. Conde, Pomales-Lebrón & Cancio (1958) have given the results obtained by electrophoretic fractionation of serum protein components during the course of experimental *Br. abortus* infection of guinea-pigs. They found an increase in the gamma globulin of the infected animals at 3–4 months after infection, and this coincided with the period of the highest agglutination titres. Awad (1959), examining sera from humans with *Br. melitensis* infection, found a rise in the gamma globulin during the acute phase of the disease. By means of chromatography Ferenčík (1959) demonstrated the presence of twenty-two amino acids in hydrolysates of brucella allergens. Nĭznánský & Krčméry (1958) have studied the catalase, urease, lipase, amylase, phosphatase and peroxidase activity of *Br. abortus*, *Br. melitensis* and *Br. suis*.

It has been found by Reusse (1958) that three out of eight strains of *Br. abortus* showed on five occasions spontaneous mutation from a mucoid to a smooth form. With the help of single-cell cultures it was shown that the S-forms isolated from mucoid cultures were not present in the inoculum. Milk from forty-five cows artificially infected with *Br. abortus* was examined culturally throughout their first lactation after challenge by Morgan & McDiarmid (1960). Excretion was intermittent, varying from 2 to 84 % of the samples. As a rule, excretion was both more consistent and abundant during the latter part of lactation, although in a few cows excretion seemed to have ceased during that period. None of the ten animals examined during the second lactation after challenge was still excreting organisms.

C. *Vaccination*

Now that the incidence of brucellosis in the U.S.A. is less and there is not the same risk of infection, Gilman & Wagner (1959) point out that it is necessary to know the optimum age for the vaccination of calves. They vaccinated with S. 19 fifteen calves at 4 months old and fifteen at 8 months old and left five calves unvaccinated as controls. All the animals were challenged by way of the conjunctival sac when 4 months pregnant. In the first group none aborted and three became infected but calved normally; in the second group one aborted and three became infected but calved normally; in the control group three aborted. There appeared to be no significant difference in the immunity produced in the two vaccinated groups. Ulbrich & Wiegand (1958) found that ingestion of colostrum during the first days of life produced a specific opsonocytophagic reaction indicative of resistance to infection which persisted up to the age of 6 months. They therefore suggest that a calf should be 6 months old before vaccination.

It has been suggested by Ullmann (1958) that high titres which are sometimes found in animals vaccinated with S. 19 are not vaccinial titres but are caused by sensitivity to small numbers of virulent *Br. abortus*. The source of this infection may be infected but non-reacting animals in the same herd. He proposed, therefore, that the serum should be tested for brucella titres before vaccination in order to detect infected calves, and that tests should be made 3 weeks later and before mating. This system was tried for a period of 4 years in a herd of 120 cows free from brucellosis, and no recurring high titres in vaccinated animals were found.

The number of cattle vaccinated with S. 19 in the Soviet Union up to the end of 1957 is given by Yuskovec (1959) as 15 million. In most of the animals its use prevented the development of brucellosis and reduced abortion in the first year of vaccination. Kolomakin & Sarsenov (1958) recorded that on 122 farms where 140 000 cows and calves were vaccinated annually with S. 19 for 3 years, abortions fell from 2735 in 1954 to three in 1957. It is stated by Ivanova (1959) that during the past 15 years eleven killed and five live brucella vaccines have been produced in the U.S.S.R. A commission of the U.S.S.R. Ministry of Agriculture at present favours S. 19 for all vaccinated cattle. Dafni (1958) has reported that good control has been achieved in Israel following the vaccination of all adult cattle with S. 19 in 1950 and the introduction of a scheme of calfhood vaccination.

It is suggested by Van Drimmelen (1957) that there is a reduced risk of local reaction if vaccination with S. 19 is given subcutaneously in the convex side of the

ear instead of into the neck. A full dose of the vaccine can be given if it is suspended in 1 ml of water. Van Drimmelen & Steyn (1958) have shown by statistical analysis that the rate of loss of viability of any particular type of freeze-dried *Brucella* vaccine can be characterized by a constant which may be termed the 'coefficient of viability loss'. This constant is believed to be of value in organizing the distribution of vaccine in widespread areas by making it possible to predict the viable count of vaccine stored at 4 °C at distribution centres. Methods under investigation at Weybridge for improving the production and keeping-qualities of S. 19 vaccine have been discussed by Hulse (1958). They include the continuous liquid culture system and freeze-dried vaccine suspended in Smith-Naylor medium in rubber-stoppered bottles.

Numerous papers refer to vaccination of laboratory animals. Haskins, Stevens & Howell (1959) have given a description of methods employing cortisone-treated mice for testing S. 19 vaccine. The susceptibility of mice to *Br. abortus* strain 544 was increased at least a thousand times after this treatment with cortisone. Spink & Anderson (1958) infected mice with S. 19. Simultaneous treatment with cortisone enhanced the infection but did not cause the severe liver necrosis and proliferation of brucella organism that had been reported previously in studies with more virulent species of brucella. Internasal immunization of guinea-pigs with S. 19 has been reported by Vershilova & Grekova (1958). Khodzhaev (1959) and Jacotot & Vallée (1959*c*) have described experimental studies of the Russian brucella strain BA, which was derived from S. 19. Jacotot & Vallée (1959*b*) have also made an experimental study in guinea-pigs of a streptomycin-dependent brucella strain derived from S. 19 with has been named strain Olitzki. This strain proved less pathogenic than S. 19 and possessed a very satisfactory immunological capacity.

A soluble-type vaccine against experimental infection in guinea-pigs has been tested by Hoag & Allen (1958). The vaccine was prepared by acid-heat extraction of *Br. abortus* strain 2308 and its metabolic by-products in Stuart's liquid leptospiral medium. Jacotot & Vallée (1958) found that white rats injected with a phenol-killed S. 19 culture possessed a strong and lasting immunity against intraperitoneal inoculation of virulent strains of *Br. abortus* or *Br. melitensis* given with mucin. The same authors (1959*a*) have confirmed their previous findings, reported in an earlier review of this series (Blackburn, 1956), that there is a relationship between the natural pathogenicity of a brucella organism and the persistence of the septicaemia it causes in guinea-pigs after subcutaneous inoculation. They advise that haemoculture be done from the third until the sixth week after inoculation. Jones, Thomson & Alton (1958*b*) have compared the immunity against brucella infection in guinea-pigs produced by three different vaccines; a killed rough-adjuvant vaccine, a killed smooth-adjuvant vaccine and a living vaccine. The immunity produced by the living vaccine was significantly greater than that produced by the killed vaccines. Challenge with four different strains of brucella did not reveal significant differences in protection afforded by rough and smooth adjuvant vaccines. Similar experiments were made on goats by the same authors (1958*a*). Prohászka (1958) has produced a strain of reduced virulence of *Br. abortus* by repeated ultraviolet irradiation. It has been found by Bekierkunst & Sulitzeanu (1958) that in mice vaccinated intra-abdominally with living B.C.G. and challenged after 2–6 weeks with virulent *Br. abortus* strain 2308, the number of brucella organisms in the spleen 7 days later was twenty-five to 300

times less than in the control animals. Markenson, Sulitzeanu & Olitzki (1959) have examined the immunizing properties of insoluble cell material derived from brucella. *Br. abortus* and *Br. suis* organisms were killed and dried with acetone, disintegrated in a sonic oscillator and centrifuged at 10000 rev/min and again at 40000 rev/min to yield two fractions. Compared in mice these fractions had a higher immunizing capacity than whole cells and the second fraction was more effective than the first.

The physical and chemical properties and agglutination content of the sera from three cattle vaccinated with S. 19 have been studied by Blum, J. (1958). He found a rise in the refractive index and an increase in the total protein especially of the gamma globulins. Bisping (1958) examined by paper electrophoresis the sera of twenty healthy cattle, twenty-four with brucellosis, and twenty vaccinated animals. He found considerable individual variation even within the normal group of healthy cattle which reduced the value of single determinations.

D. *General*

The interactions between the mononuclear phagocytes of normal and immune guinea-pigs and strains of *Br. abortus* of different virulence have been studied by Braun, Pomales-Lebrón & Stinebring (1958). They found differences in the intracellular multiplication and the rate of ingestion by normal monocytes, between S and R forms of *Br. abortus*, and a modification of these differences in immune monocytes. S. 19 showed an apparently unique behaviour in normal monocytes. Stinebring & Kessel (1959) have accomplished continuous growth of smooth forms of virulent *Br. abortus* organisms within mononuclear cells of guinea-pigs, by periodic transfer from tissue culture to tissue culture. Comparable continuous growth could not be accomplished with rat-monocyte cultures. These results agree with what is known about the fate *in vivo* of the organisms in these two animals. Guinea-pigs are susceptible to small numbers of smooth brucellae whereas rats seem to possess a higher resistance. Freeman & Vana (1958) found that small numbers of brucella organisms were capable of infecting a tissue culture of mononuclear cells from normal guinea-pigs, and of multiplying rapidly. No lag-phase was observed even when the inoculum was as low as eighty cells taken from a culture in the maximum stationary phase. They found no consistent correlation between the virulence of the organisms used and the growth rate. Holland & Picket (1958) showed by microscopic and cultural procedures that *Br. suis*, *Br. abortus* and *Br. melitensis* multiplied extensively within normal rat, mouse and guinea-pig monocytes maintained *in vitro* in cell cultures for 3 days. In contrast, monocytes derived from animals previously infected with smooth brucellae greatly restricted the intracellular growth of smooth and non-smooth forms and of S. 19.

Parnas, Blitek, Kozicka & Zuber (1957) inoculated chick embryos into the yolk sac with varying numbers of brucella organisms of S. 19, strain BA and 24. All these strains caused pathogenic changes. On the ninth day fourteen of 350 embryos inoculated with S. 19 were still viable, but all of 700 inoculated with the other two strains were dead by the seventh day. Kamal (1959*a*) found that in 6-day-old chick embryos brucella infection was always induced by inoculation of four organisms, and sometimes by two, and that the older the embryo the longer was the time between inoculation and death. He made a quantitative determination of the distribution of

brucella organisms throughout the developing egg. The same author (1959*b*) has reported that repeated egg-to-egg transfers did not alter the colonial form of growth of *Br. abortus* nor its ability to agglutinate, but increased its virulence for chick embryos and mice. He made a histological study of the response of the different tissues of the embryo to bacterial invasion. Sulitzeanu (1959) has studied the fate of radio-iodinated *Br. abortus* injected into mice. He found that after injection of acetone-killed organisms, the spleen and liver of non-immune mice collected a higher amount than the corresponding organs of immune mice. A considerable proportion of the injected organisms was destroyed and the breakdown products excreted during the first 6 h after inoculation. Gerhardt (1958) has reviewed the literature on the nutrition of brucellae.

Referring to the incidence of brucellosis, Marr & Williams (1958) give details of an investigation made during the years 1954–6 which showed that 25% of the dairy herds in the northern counties of Scotland gave a positive milk ring test (M.R.T.). *Br. abortus* was recovered from approximately 25% of the M.R.T. positive milk bulk samples. Examination of individual animals showed that a high proportion of cows with positive M.R.T. and positive vaginal mucus tests were excreting brucella organisms in their milk. Seventy-eight strains of brucella were isolated during the investigation but none resembled S. 19. From a survey made in the years 1956–8 Alton (1959) estimated that there were 300 to 400 reactors amongst the cattle on the Island of Malta, an average of 3·5 animals per infected herd.

The pathogenesis of experimental brucellosis in the pregnant cow has been studied by Payne (1959). He used a culture of *Br. abortus* strain 544 (McEwen) containing 1500×10^6 organisms per ml and dropped 0·1 ml into the conjunctival sac. The progress of the infection was followed in eleven cows. Infection appeared to drain from the conjunctiva into the parotid and other lymph nodes of the head, and then to other lymphoid tissues such as the spleen and to the iliac and supermammary lymph nodes. A mild acute lymphadenitis developed in each node, and hyperplasia of the dense cortical lymphatic tissue occurred. Later, large numbers of plasma cells were formed in the medullary cords and the adjacent sinuses underwent severe catarrhal change. The earliest lesion in the uterus appeared to be inflammation in the connective tissue between the uterine glands, in which purulent exudate was soon formed. This exudate appeared to be the origin of the heavily infected uterine exudate which is so characteristic of the disease. At or near abortion infection spread into the depths of the cotyledons and the blood vessels soon became occluded. Eventually whole villi were destroyed and replaced by pus. There was some evidence of a toxic reaction in such organs as the kidney which were distant from the sites of infection. Moulton & Meyer (1958) have studied the pathogenisis of *Br. suis* infection in guinea-pigs. They give a very clear description of the gross and microscopic pathology of the spleen, liver, testis and joints at different times after infection. The organisms were seen predominantly in polymorphs and it appeared that these cells were primarily responsible for transporting the bacteria to different parts of the body. Jacob (1959) has given the results of the examination of twenty-five udders and their associated lymph glands from animals serologically positive both in blood and milk. In twenty-three of the animals *Br. abortus* was recovered from the milk or the udder tissue by culture and in two by animal inoculation. Macroscopically no lesions could be detected, but histo-

logically seventy-eight quarters showed granulomatous formation with intralobular proliferative mastitis and the regional lymph nodes showed mainly a proliferative reticular reaction. Some of the changes resembled those of indurative mastitis of various origins, but the author considers that the granulomatous formation was specific and its presence corresponded approximately with the bacteriological findings.

Numerous papers have been published on the subject of human brucellosis. Held, Bauer & West (1958) have found that coincident with the progress of eradication of brucellosis in cattle there has been a significant reduction in human cases. From 1950 to 1956 the incidence in humans in Minnesota declined markedly despite a high increase in cattle population. Studies in Iowa, Minnesota and Wisconsin by Helvig (1959) have shown a correlation between incidence of human and animal brucellosis. During the 10-year period 1947–56 the States of the U.S.A. reported to the National Office of Vital Statistics a total of 31132 cases of human brucellosis. The annual incidence of reported cases fell gradually from 6321 in 1947 to 1100 in 1956. Wundt (1959) has stated that a survey in Würtemberg has shown that the highest incidence of human brucellosis due to *Br. abortus* occurred in districts with the largest cattle population, and the highest incidence due to *Br. melitensis* in districts with the largest sheep population. He referred, however, to several cases of human brucellosis due to *Br. melitensis* infection in dairy cows. A study of 121 probable cases of human brucellosis in Victoria was made by Wilson, M. M. (1957). Of these 121 people thirty-six were dairy farmers, one a milkman and a further forty were in occupations involving the handling of cattle or cattle products. The epidemiological findings suggested that infection occurred through handling infected cows, though milk-borne infection may have played a part. A paper by Nussbaum (1958) gives the incidence of human brucellosis in the Bernese Mittelland during the years 1955–56 as 1 in 10000 of the population (fifty-four cases). Detailed case histories of forty-one of these showed that twenty-four were engaged in agriculture. Infection with *Br. suis* of a 4-year-old boy in Eire has been reported by Williams, Entwhistle, Masters & Woods (1957). Papers by Sadusk & Born (1957) and Spink (1957) refer to accidental infection with S. 19 vaccine. Linsert (1958) found that the sera of fifty-one of 104 veterinary surgeons examined were positive to the slow agglutination, complement-fixation and flocculation tests for *Br. abortus*. Serological tests on sera taken from veterinary personnel in Cracow by Lutyński (1958) showed 99 % to be infected with brucellosis, and of these 5·9 % were clinical cases. Davies (1957) has described the clinical and laboratory features of chronic brucellosis which occurred in a rural practice in Wales. It has been found by Kerr *et al.* (1958) that in Northern Ireland a significant number of herds contained one or more vaccinated cows which disseminated infection via the udder, a finding of importance for the public health authorities, especially since milk from many such herds is sold unpasteurized. Schaal (1958) has isolated brucella from the milk of twenty-nine out of eighty infected cows sent for slaughter, and he is of the opinion that the existing law in Germany which permits the use of udders for sausage-making when infected with brucellosis should be changed. Using a selective medium, Neri & Odasso (1957) studied the distribution of brucella organisms during the laboratory manufacture of butter from artificially infected milk. It was found that the organisms, which were concentrated in the cream, passed to a large extent into the buttermilk during manufacture. Brucella organisms survived

for 23 days in butter made from artificially infected milk stored at 4 °C. Guerra (1957) found that in cream samples inoculated with *Br. melitensis* or *Br. abortus* and stored at 4 °C the presence of brucella organisms could be demonstrated at 4 and 6 weeks respectively. Olitzki, Sulitzeanu, Arnan & Rasooly (1960) have discussed observations they have made on men vaccinated with a streptomycin-dependent strain of brucella. Strong post-vaccinial reactions were observed in fourteen of eighty individuals vaccinated. Four of them were known to be infected and the rest were apparently suffering from a latent infection at the time of vaccination. Parnas, Chodkowski, Lazuga & Kozicka (1958) have described experiments on the choice of a strain suitable as a live vaccine for human beings. They considered that strain PD, a strain with properties intermediate between S. 19 and a strain 45/20, was suitable for further study. The subject of the transmission of brucellosis to man has been reviewed by Renoux (1959) who has given a list of 372 references.

A few papers dealing with the treatment of brucellosis have been published recently. Bauer (1959) has found that of numerous antibiotics and chemical substances tested for anti-brucella action in mice and hamsters in the last 4 years, streptomycin and 'Berenil' (4,4-diamidino-diazobenzol) proved most satisfactory. In tests on cows secreting organisms in the milk, 'Berenil' was of little use, though streptomycin and tetracycline, especially the former, were effective. With combined cortisone and streptomycin given over a 27-day period to four cows, brucella disappeared from the milk for more than 2½ years in one cow, for more than a year in two others, but only for 6 weeks in the fourth. A new drug 'Pecudin' (N^1-dichloracetyl-N^1-phenyl-semicarbazide) has been used in the treatment and prophylaxis of bovine brucellosis. Pilz (1958) concluded that 'Pecudin' will prevent brucellosis in cattle during the period of administration but will not cure infected animals, and Rosenberger & Grunert (1958) found that 'Pecudin' administered regularly at the rate of 5 g per cow per day had a definite prophylactic effect against brucellosis in artificially infected animals, but the treatment did not prevent brucella organisms from appearing in the milk.

There is a continued interest in animals that may act as reservoirs or carriers of brucellosis. Corner & Connel (1958) examined serum samples from 343 bison, 124 moose and 221 elk in Elk Island National Park, Alberta, Canada, which were slaughtered in the winter of 1956–57. Agglutination tests showed that about 42 % of the bison and 13 % of the elk were reactors. None of the slaughtered moose was a reactor, possibly because brucellosis is more fatal in this animal. It is thought that the elk may play a part in the spread of brucellosis, including transmission to domestic cattle outside the National Parks. Gentile (1957) demonstrated *Br. abortus* bacteriologically and biologically in aborted foetuses, foetal membranes and blood of buffaloes which aborted at the sixth to eighth month of pregnancy. Youatt & Fay (1959) have found it possible to infect the white-tailed deer experimentally with *Br. abortus*, but they are not able to say whether this animal is as susceptible as cattle in the natural state. In work by Guerra & Benelli (1959) subcutaneous injection of *Br. melitensis* and *Br. abortus* did not cause disease in hares. The role of cats as carriers of brucellosis has been studied by Smutný (1958). He fed ten cats with a virulent 48-h culture suspended in milk, and all the animals reacted with an increase in the agglutination titre and by faecal excretion of viable brucella organisms for varying periods up to

the twelfth day after ingestion. Brucella organisms were demonstrated by Harnach (1957) in 11 % of the flies caught in a cowshed where there were abortions and in 13 % of those caught on the manure heap of the same farm. Karásek (1958) demonstrated viable brucella organisms on the body surface of mosquitoes (*Culex pipiens*) up to 36 h, in the digestive tract up to 72 h, and in the faeces up to 48 h after ingestion of experimentally infected blood.

With reference to the spread of brucellosis within herds, Kerr & Rankin (1959) have confirmed experimentally that the milking machine can spread brucella infection from one infected udder to another. Wellman & Liebke (1959) have made experimental studies on the possible transmission of brucellosis by bullocks. Eight bullocks were inoculated by various routes with *Br. abortus*. Positive agglutination titres were present in some of the bullocks 14 days after infection but they gave negative tests after 6–14 weeks. In six animals it was possible to detect the organisms in the blood at intervals even though the titre had become negative. Transmission of infection to pregnant heifers by contact was not detected.

E. *Control and eradication*

It has been stated by Gregory (1958) that in Australia calfhood vaccination with S. 19 has become an established practice during the last 10 years, and it is generally accepted that major efforts in control should be directed towards this type of vaccination. He is of the opinion that throughout the Australian Commonwealth agglutination tests should conform to internationally recommended checks for accuracy. He points out that the agglutination test can be negative though organisms can be cultured from the blood or milk, and other fallacious results can be obtained with pregnant and aborting animals and with others because of incomplete antibodies. The progress of eradication in Tasmania has been described by Clark (1958). Two methods are being used, vaccination in areas of high incidence, and test and slaughter where incidence is low. Of herds released from quarantine 6 % have recurrences within 12 months and these are attributed to hidden carriers. Only 0·3 % of cattle now remain infected whereas the initial incidence was 2·8 %. Discussing the eradication of bovine brucellosis in the U.S.A., Mingle (1959*a*) has explained that since 1947 the co-operative brucellosis programme has been conducted under uniform methods and rules recommended and approved by the livestock industry, livestock sanitary agencies and the Department of Agriculture. Procedures consist essentially of (*a*) test and slaughter without calfhood vaccination, (*b*) test, calfhood vaccination and the temporary retention of reactors, and (*c*) calfhood vaccination without testing the herd. The first is the ideal method and the other two merely steps to reduce infection rates to a level where reactors can be removed without serious economic loss to the owner. Mingle (1959*b*) has pointed out that infection has been reduced from 11·4 % of all cattle blood tested in 1954 to only 1·5 % in 1959, and the latter figure is even more remarkable when it is remembered that now the majority of blood tests are done on herds known by the milk ring test to be infected.

Cameron (1959) has reported on the use of the milk ring test, the whey test and blood test for eradicating brucellosis from Mavin County, California, where the herds are large and on hill pastures, replacements being reared, as a rule, on the ranch. Non-dairy herds were blood-tested and dairy herds ring tested. Lactating animals in herds

in which the ring test was doubtfully positive were whey tested and dry cows blood tested, and reactors were slaughtered. This method has reduced herd infection from 30·4% to less than 3·8% and cattle infection from about 1% to less than 0·1%. Heuner (1958), discussing the regulations concerning the control of brucellosis in cattle in Federal Germany, has stressed that differences in different regions can give rise to misunderstandings and seriously hamper the eradication of the disease. Pillon (1958), in referring to the control of brucellosis in Denmark, has explained that the legislation is similar to that for the control of tuberculosis. Owners are required to register herds for sale for export, obtaining a certificate from a veterinary surgeon stating that the animals are free from brucellosis. When reactors are found in a herd they are isolated and eliminated as soon as possible.

F. *Conclusions*

It would appear that the term 'Contagious Abortion' in cattle has ceased to have much meaning in countries where there is calfhood vaccination with S. 19. In such countries the main problem is concerned not so much with the aborting animal as with the infected animal, more especially the animal giving infected milk, and the consequent risk to the human consumer. Year by year there is an increasing awareness of this problem, and the only sure remedy is the eradication of the disease. The milk ring test has now been generally accepted as a screening test in any scheme for the eradication of brucellosis. This test done on a sample of bulk milk from a herd will show in the majority of instances whether or not the milking herd contains any reactors, and further tests on blood serum or milk from individual animals will reveal these reactors. Provided that there has been no vaccination of adult cattle with live vaccine, reactors can be considered as infected animals.

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